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CD4+ cells in human ejaculates

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Using flow cytometry, we studied the expression of the CD4 antigen within the different cells present in human ejaculate, both in spermatozoa and round cells. In all, 20 samples of semen were obtained from fertile males; in 11 of these, we detected the presence of leukocytes, using the peroxidase test. Swim-up was performed for the analysis of the spermatozoa. From our results it may be concluded that there is no expression of the CD4 antigen on the surface of human spermatozoa or on CD45– ejaculate cells (epithelial and germinal cells). However, we did detect the presence of the CD4 antigen on the surface of the leukocyte cells (CD45+). A better characterization of these CD45+ cells made it apparent that the CD4+ cells of ejaculate are composed of T lymphocytes (helper/inducer T lymphocytes) and monocytes. Thus we may conclude that human spermatozoa do not express the CD4 antigen, the cell surface receptor for human immunodeficiency virus. However, we did detect CD4+ T lymphocytes and CD4+ monocytes in semen.

Key words: flow cytometry/human immunodeficiency virus/semen

Introduction

Various studies have shown the importance of semen as one of the main agents for the transmission of human immunodeficiency virus (HIV). HIV is detectable in seminal leukocytes and in cell-free seminal plasma (Zagury *et al.*, 1984; Olsen and Shields, 1984; Ho *et al.*, 1984; Dalgleish *et al.*, 1984; Gartner *et al.*, 1986; Anderson and Hill, 1987; Pudney *et al.*, 1992; Meltzer and Gendelman, 1992; Pearce-Pratt and Phillips, 1993; Leach *et al.*, 1993). Nevertheless, it has not been established just what is the mechanism by which the virus enters the ejaculate and whether spermatozoa or immature germinal cells can be infected (Witkin *et al.*, 1975; Basgara *et al.*, 1988; Baccetti *et al.*, 1991; Mermin *et al.*, 1991; Ilaria *et al.*, 1992; Hamed *et al.*, 1993; Ablin, 1993; Pearce-Pratt and Phillips, 1993).

Since CD4 is the cellular receptor of HIV and as it has been speculated that the transmission of HIV in semen is influenced by the expression of the CD4 receptor by the spermatozoa, we decided to study the expression of the CD4 antigen in different cells present in human ejaculate.

Materials and methods

Processing of semen samples

We studied 20 semen samples from fertile prevasectomic males (sperm concentration (mean ± SD) 166.1 ± 70.9×10^6 spermatozoa/ml; progressive motility 65.1 ± 9.5%). The samples were obtained by masturbation, after 3 days of sexual abstinence, into sterile plastic containers. HIV serology for the 20 patients and the cultures obtained from all the ejaculates proved to be negative. Two aliquots were obtained from each ejaculate: one of which was prepared for swim-up; and the other was used for the analysis of the seminal leukocytes. The swim-up was performed by adding 3 ml of Roswell Park Memorial Institute (RPMI) culture medium (Flow Laboratories, Irvine, UK) to 1 ml of semen and the mixture was centrifuged at 300 *g* for 8 min. The supernatant was immediately removed, and 1 ml of the medium was very slowly added to the pellet, which was then incubated at 37°C for 40 min. The motile spermatozoa migrated to the medium and they were then collected and counted in a Neubauer chamber.

Expression of antigens by spermatozoa

Subsequently 50 ml of each spermatozoa sample was incubated in darkness at room temperature for 30 min, with 10 ml of anti-CD45 monoclonal antibody directly conjugated to fluorescein isothiocyanate (Anti-HLE-1 FITC; Becton Dickinson; Mountain View, CA, USA) as the negative control, and also with 10 ml of anti-CD59 monoclonal antibody (CLB-CD59, clone MEN 43, Menarini Diagnostics; Barcelona, Spain), as the positive control (Rooney *et al.*, 1992; D'Cruz and Haas, 1993; Simpson and Holmes, 1994) by indirect immunofluorescence; and finally, another 50 ml of each sample was incubated with 10 ml of anti-CD4 monoclonal antibody directly conjugated with FITC (Anti-Leu-3 FITC; Becton Dickinson). After the incubation period, two washes with phosphate-buffered saline (PBS) were carried out and measurements were taken in the FACscan IV flow cytometer (Becton Dickinson; Figure 1). In the case of CD59 monoclonal antibody, the cells were then washed twice and reincubated for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab')_2$ (Chemicon, Temecula, CA, USA). After two washes, monoclonal antibody (MAb) binding to the cell preparations was assessed by flow cytometry.

Expression of CD4 antigen by the round cells

To study the seminal leukocytes, we used a direct immunofluorescence technique of single and double staining of monoclonal antibodies. From the total aliquot of semen, the round cells and positive peroxidase leukocytes were counted in an optical microscope. After this, the

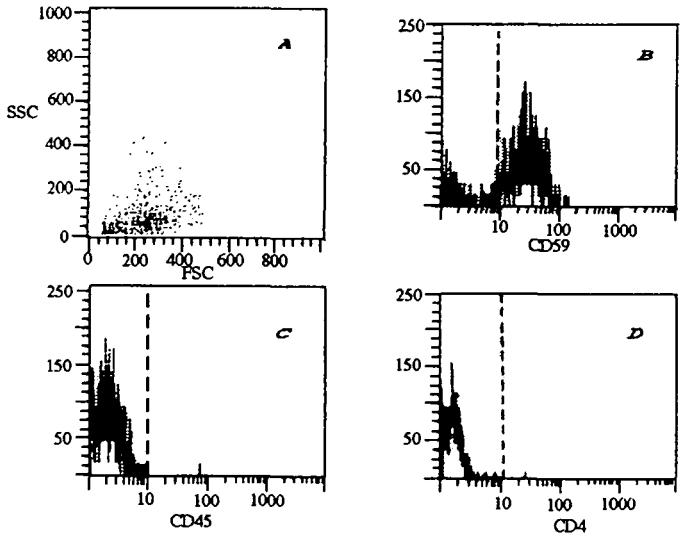

Figure 1. Study of the expression of the CD4 antigen by the swim-up spermatozoa of fertile ejaculate: (**A**) Graphic representation of a swim-up sample (no. 12) by flow cytometry, by means of FSC (cell size) and SSC (granularity) detectors. Capture of anti-CD59 monoclonal antibodies (**B**); anti-CD45 (**C**); and anti-CD4 (**D**) by the sample spermatozoa. The spermatozoa reacted with the anti-CD59 monoclonal antibodies in >90% of cases, but did not react with the anti-CD45 or anti-CD4 antibodies. In histograms (**B**), (**C**) and (**D**), the *y* axis represents number of cells and the *x* axis the log of fluorescence intensity.

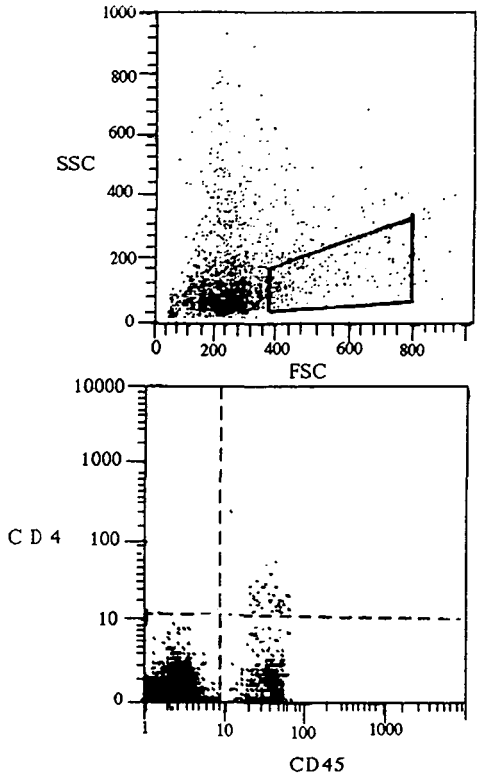

Figure 2. Analysis by flow cytometry of an ejaculate (no. 12). The upper figure indicates the window for round cells, with cell size represented on the *x* axis and granularity on the *y* axis. The lower figure represents the leukocyte population (CD45+) included in the group of round cells (which in this case amounted to 35%) and the proportion of the former that co-expressed the CD4 antigen.

sample was diluted with a volume of PBS equal to that of the aliquot of semen and centrifuged for 5 min at 400 *g*. The pellet was resuspended in 0.5 ml of PBS and finally shaken in order to obtain a homogeneous cellular solution. Following the method of Parker *et al.* (1990) and according to the rapid method of Hoffman *et al.* (1980) in peripheral blood, and its application in liquids of biological nature, as suggested by Castilla *et al.* (1990): 50 ml of semen sample were mixed with 10 ml of an initial monoclonal antibody conjugated with phycoerythrine (PE). The sample was shaken and incubated for 30 min at room temperature in darkness. Consequently it was washed twice with PBS, adding 3 ml of this solution, and then shaken and centrifuged at 400 *g* for 5 min. Finally, the cells were decanted and resuspended in 200 ml of 1% paraformaldehyde, before analysis of the seminal leukocytes by means of the FAC-scan IV flow cytometer (Becton Dickinson; Figure 2). The different combinations of monoclonal antibodies used are summarized in Table I.

In this way, we used the total semen samples to study spermatozoa (Figure 3) by direct and indirect fluorescence techniques of double staining of monoclonal antibodies (Table I), following the same procedures indicated for the seminal leukocytes.

Flow cytometry analysis

The identification of spermatozoa and round cells was carried out in the FAC-scan IV flow cytometer (Becton Dickinson) by distributing the cells by size (identified by the Forward Scatter detector) and granularity or cytoplasmatic complexity (analysed by the Side Scatter detector), recognizing, as noted by Haas and Cunningham (1984), the existence of a small percentage of error (~5%) in the design of the spermatozoa window, in which some round cells may be included, and vice versa (Figures 2 and 3); in the same way, and using the fluorescence detectors (FL1 and FL2), an analysis was performed of the populations of leukocytes, monocytes and lymphocytes, in each case to determine and quantify the percentage of CD4+ cells existing in these subpopulations.

Results

Analysis by flow cytometry of the CD4+ cells in human ejaculate revealed the absence of the expression of the CD4 antigen on the surface of spermatozoa (Figures 1 and 2), apparent both in the swim-up and in the analysis of total semen carried out for each ejaculate. No marker was used for immature germ cells but CD45– in the round cell window did not express CD4 antigen. However, CD4 antigen was detected on the surface of the leukocyte cells (CD45+) (Figure 3).

In 11 of the ejaculates, the presence of leukocytes was detected using the peroxidase test (World Health Organization, 1992). Individual characteristics of the 20 semen samples are shown in Table II. CD45+ cells were observed in eight (40%) of the ejaculates with a concentration (mean ± SD) of 238 ± 409×10^3/ml; the values ranged between 0–1700×10^3/ml. Improved characterization of these CD45+ cells allowed us to determine that the CD4+ ejaculate cells were composed of T lymphocytes, with a concentration of 9 ± 17×10^3/ml (helper/inducer T lymphocytes) and monocytes with a concentration of 157 ± 289×10^3/ml.

The discrepancy in the number of ejaculates in which leukocytes were observed found by the two different methods

Table I. Cellular populations characterized in our study

CD	Monoclonal antibody	Specificity
CD45 FITC	Anti-HLE-1	Pan-leukocyte (negative control to spermatozoa)
IgG_1 FITC + IgG_{2a} PE	Simultaneous control	Negative control to leukocytes
CD45 FITC + CD4 PE	Anti-HLE-1 + anti-Leu-3a	CD4+ leukocytes
CD4 FITC + CD14 PE	Anti-Leu-3a + anti-Leu-M3	CD4+ monocytes
CD4 FITC + CD3 PE	Anti-Leu-3a + anti-Leu-4	CD4+ lymphocytes (helper/inducer)
CD59 Anti-protectin	Positive control to spermatozoa	
CD59 FITC + CD4 PE	Anti-protectin + anti-Leu-3a	$CD4^+$ spermatozoa

Ig = immunoglobulin; FITC = fluorescein isothiocyanate; PE = phycoerythrine.

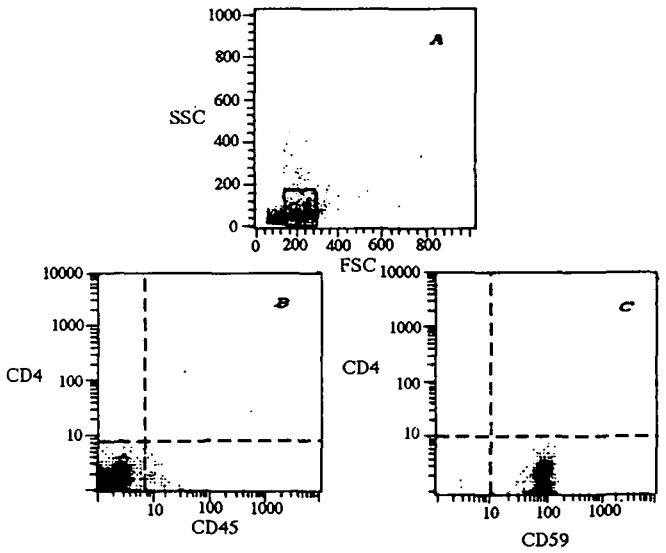

Figure 3. Analysis by flow cytometry of an ejaculate (no. 12), showing cell size on the *x* axis and granularity on the *y* axis. (**a**) Gating of the sample spermatozoa. (**b**) Analysis of the expression of the anti-CD45 and anti-CD4 monoclonal antibodies by the selected spermatozoa. No capture was observed by these cells. (**c**) Analysis of the expression of the anti-CD59 and anti-CD4 monoclonal antibodies by the gated spermatozoa. We observed the expression of CD59 antigen on >90% of the spermatozoa (positive control), but there was no capture of the anti-CD4 monoclonal antibody by these cells.

may be due to the fact that flow cytometry is less sensitive than the peroxidase test and probably requires a minimum number of 10^5 seminal leukocytes/ml.

Discussion

Recent studies have demonstrated some of the transmission mechanisms of HIV in semen. Various researchers (Witkin *et al.*, 1975; Ilaria *et al.*, 1992; Hamed *et al.*, 1993) have detected the activity of inverse transcriptase in human spermatozoa, while others have revealed the presence of viral particles in seminal fluid (Borzy *et al.*, 1988; Ablin, 1993). Nevertheless, there are opposing opinions (Semprini *et al.*, 1992; Anderson *et al.*, 1992) drawing attention to the absence of a clear mechanism by which HIV enters the spermatozoa, implicating the seminal fluid in the transmission of auto-immune disease (AIDS).

There is important evidence to suggest that the sexual transmission of AIDS is effected by means of the seminal leukocytes (Zagury *et al.*, 1984; Olsen and Shields, 1984; Dagleish *et al.*, 1984; Gartner *et al.*, 1986; Anderson and Hill, 1987; Meltzer and Gendelman, 1992) that may infect the epithelium of the male genital tract, the semen being a potential transmitter and reservoir of HIV (Pearce-Pratt and Phillips, 1993). Zagury *et al.* (1984) have also reviewed innovatory aspects regarding the entrance of the virus into seminal leukocytes, as a vector of infection, demonstrating that the expression of the HIV antigens in these cells is transitory, with no activity being detected either of the inverse transcriptase or of the viral antigens in seminal leukocyte cultures after a period of 12 days.

Another consideration is the fact that the main objective of the virus is a cellular subpopulation that expresses a glycoprotein termed CD4 on its surface, which adheres to the protein covering of HIV. Some authors have related the transmission and reproduction of the virus in semen to the level of CD4+ leukocytes (Ho *et al.*, 1984; Anderson and Hill, 1987), and have even found that a diminution of these cells in semen increases the risk of certain opportunist infections (Wolff and Anderson, 1988b), such as CMV (Leach *et al.*, 1993). Since the adherence of the virus to the CD4+ cells is the standard method described as being essential for the virus to invade and hence produce the clinical symptoms of AIDS, some researchers suggest that the entry of the virus into human spermatozoa may occur by means of the CD4 receptor, a theory upon which opinions are divided (Ashida and Scofield, 1987; Wolff *et al.*, 1988; Crittenden *et al.*, 1992).

As has been observed by Wolff *et al.* (1988) and Nuovo *et al.* (1994), our results show that neither the spermatozoa nor the immature germinal cells present in the ejaculate express the CD4 antigen, the cellular surface receptor for HIV. Nevertheless, we are not able to claim that semen does not constitute one of the means of transmission of this infection because in the ejaculates of fertile individuals, concurring with the other authors (Wolff and Anderson, 1988a), we detected CD4+ lymphocytes and CD4+ monocytes, which are target cells for the advance of this disease. Our results confirm the necessity for the caution in the selection of semen donors that must be exercised by any human reproduction laboratory, as has previously been observed by other authors (Anderson and Hill, 1987), as, although human spermatozoa do not appear to possess the surface receptor for the virus, we have detected the presence of CD4+ leukocytes in the ejaculates studied.

Moreover, some authors (Basarga *et al.*, 1988; Baccetti *et al.*, 1991; Scofield *et al.*, 1992) have detected HIV in the

Table II. Individual characteristics of semen samples

Sample no.	No spermatozoa $\times 10^6$/ml	Percentage motility	No. spermatozoa $\times 10^6$/ml after swim-up	No. round cells $\times 10^3$/ml	No. positive peroxidase cells $\times 10^3$/ml	Percentage CD45+ round cells	CD4+ spermatozoa
1	120	56	16	200	0	0	0
2	236	76	34	1000	0	0	0
3	198	68	45	5000	0	0	0
4	150	55	23	4000	1000	13.5	0
5	125	80	18	3500	500	15.4	0
6	99	69	19	2700	500	17.7	0
7	80	54	9	1000	0	0	0
8	190	58	14	500	50	0	0
9	298	55	34	100	0	0	0
10	321	51	10	3200	500	17	0
11	201	64	13	200	50	0	0
12	167	81	21	5000	1500	34	0
13	65	77	8	100	50	0	0
14	210	66	23	1500	300	18.5	0
15	110	67	17	800	0	0	0
16	222	65	9	750	0	0	0
17	129	78	19	2000	250	15.4	0
18	200	67	13	100	0	0	0
19	78	56	7	3500	100	10.7	0
20	123	59	18	200	0	0	0

spermatozoa, although its entry mechanism remains unknown because, as we show, this latter does not express the CD4 antigen. However, we cannot discount the possibility that the entry of the germinal cell may occur during another phase of spermatogenesis and by means of a receptor other than CD4. Thus, Nuovo *et al.* (1994) have found that HIV-1 selectively infects the spermatogonia, the spermatocyte and, to a lesser degree, the spermatid. Significantly, the CD4 receptor has not been detected in the spermatogonia, which suggests that the human immunodeficiency virus may join and become internalized within the spermatogonia by means of other receptors such as galactosyl-ceramide, a glycolipid that acts as a receptor for the infection of different cell types (Harouse *et al.*, 1991).

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