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Leukocytic Infiltration into the Human Ejaculate and Its Association with Semen Quality, Oxidative Stress, and Sperm Function

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ABSTRACT: Immunocytochemical techniques have been used to monitor the size and composition of the leukocyte population in unfractionated human semen samples and sperm populations generated by Percoll® gradient centrifugation. The characteristics of the leukocyte population have then been related to the quality of the semen profile, the production of reactive oxygen species, and the functional competence of the spermatozoa. A majority (97%) of the ejaculates examined contained leukocytes, and in 82.4% the major cell type was the granulocyte. Small numbers of T cells, B cells, and monocytes/macrophages could also be found in 62%, 43%, and 21% of samples, respectively, and patients were occasionally identified in whom one of these cell types became the predominant leukocyte species. Although a subpopulation of patients was identified in whom the infiltration of multiple leukocyte species was positively correlated with the concentrations of spermatozoa and precursor germ cells in semen, in general, the presence of leukocytes, to the point of leukocytospermia, did not significantly influence any component of the semen profile. Similarly, the fertilizing potential of the washed spermatozoa, as assessed by *in vitro* tests of the acrosome reaction and sperm–oocyte fusion, was not correlated with the concentration of seminal leukocytes. In contrast, the carryover of leukocytes into the washed sperm preparations profoundly influenced the fertilizing potential of the spermatozoa via mechanisms that were associated with the production of reactive oxygen species. These results have implications for the diagnostic significance of leukocyte contamination in the context of male infertility and assisted conception.

Key words: Leukocytes, reactive oxygen species, sperm function.
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Leukocytic infiltration into the human ejaculate is thought to be a clinically significant factor in both the sexual transmission of retroviruses (Anderson and Hill, 1987; Anderson, 1992) and the etiology of infertility (Wolff and Anderson, 1988; Barratt et al, 1990; Wolff et al, 1990). Despite the significance of these cells, the composition of the leukocyte subsets in human semen has received little attention, and the relationship between the presence of such cells and the fertilizing potential of the ejaculate is still highly controversial. In terms of leukocyte composition, there is evidence to suggest that granulocytes, macrophages, and lymphocytes can all be found in a majority of human semen samples although the predominant cell type has been variously identified as lymphocytes (Couture et al, 1976) or polymorphs (El-Demiry et al, 1986a,b; Wolff and Anderson, 1988; Tomlinson et al, 1992). In terms of fertility, a negative impact of leukocytic infiltration was indicated by Wolff and Anderson (1988), who found higher numbers of granulocytes, monocytes/macrophages, and T-lymphocytes in the semen of infertile patients compared with fertile controls. In a separate study, Wolff et al (1990) also observed powerful associations between the incidence of leukocytospermia and abnormalities in sperm number and motility. In contrast, neither Bassol et al (1990) nor Aitken et al (1992c) could detect any significant difference in leukocyte counts between groups of patients exhibiting either a normal semen profile or oligozoospermia, and Tomlinson et al (1992) even found oligozoospermia to be associated with a significant decline in leukocyte numbers. Similarly, El-Demiry et al (1986b) could find no significant relationship between leukocyte number and the incidence of subsequent pregnancy and, in general, recorded higher concentrations of leukocytes in the ejaculates of fertile donors than infertile patients. Moreover, Tomlinson et al (1992) suggested that leukocytes might play a positive role in shaping the semen profile by phagocytosing morphologically abnormal spermatozoa.

In light of these conflicting studies there is currently no clinical consensus as to the nature and significance of the leukocyte population in the human semen samples. Thus, the present study set out to address this area, using definitive immunocytochemical techniques to generate data on the size and composition of the leukocyte population

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that could then be related to the quality of the semen profile and the fertilizing potential of the spermatozoa.

Materials and Methods

Spermatozoa

Human spermatozoa were provided by a cohort of 120 patients attending an infertility clinic; they were in normal health at the time of analysis and their female partners were free of any major reproductive abnormalities on the basis of a normal history and examination, normal luteal phase progesterone levels, and a normal laparoscopy, including the demonstration of tubal patency (Aitken et al, 1991). Semen quality was assessed in terms of sperm concentration, total sperm count, motility, and morphology, using the guidelines laid down by the World Health Organization (WHO) (World Health Organization, 1992). The movement characteristics of the spermatozoa were assessed using a Hamilton Thorne HT-M2030 Motility Analyzer (Hamilton Thorne Research, Danvers, Massachusetts), as described by Aitken et al (1992a). The semen profiles exhibited by this group of patients are indicated in Table 1. Of the 120 samples examined, most were normozoospermic; however, 12 specimens exhibited oligozoospermia (<20 × 10^6 spermatozoa/ml), 22 were asthenozoospermic (<40% motility), 37 were mildly teratozoospermic (20–40% normal morphology), and in a further 18 specimens the teratozoospermia was more severe (<20% normal).

Leukocytes

Leukocytes were identified using the immunocytochemical procedures described by Aitken and West (1990) and Aitken et al (1992c). A 50-μl aliquot of semen was diluted with 1 ml of Dulbecco's phosphate-buffered saline (PBS; Flow Laboratories, Irvine, Scotland), centrifuged at 500 × *g* for 5 minutes, and then resuspended in 50 μl of PBS. Aliquots (5 μl) of each cell suspension were then dried down onto the wells of a Hendley slide (C. A. Hendley, Essex, England), wrapped in aluminum foil, and stored at −70° C until analyzed. Each Hendley slide contained a duplicate pair of positive control wells in which 5-μl aliquots of a peripheral leukocyte suspension had been air dried; negative control slides omitted the primary antibody.

Prior to staining, the slides were allowed to come to room temperature and were then fixed in acetone: methanol: formaldehyde (95:95:10) for 90 seconds and washed with Tris-buffered saline (0.05 M Tris and 0.15 M NaCl, pH 7.6). Five μl of a monoclonal antibody, at a dilution of 1:10 (approximately 2 μg IgG/ml), was then spread evenly over each well. The slides were incubated for 30 minutes in a humidified chamber at room temperature before being washed two times in Tris-buffered saline. The size of the total leukocyte population was assessed using a monoclonal antibody directed against the common leukocyte antigen (CD 45; Scottish Antibodies Production Unit, Carluke, Scotland). Nucleated round cells that did not stain with this monoclonal antibody and exhibited the appropriate morphology (World Health Organization, 1992) were classified as precursor germ cells. Further differentiation of the leukocyte subsets was achieved on the basis of the cells' morphology and their cross-reactivity with the following antibodies: anti-TG1 for granulocytes (Scottish Antibodies Production Unit), anti-Leu 4 for T-lymphocytes, anti-M3 for macrophages/monocytes (Becton Dickinson, Cowley, Oxford, England), and anti-Pan B for B-lymphocytes (DAKO Ltd., High Wycombe, England).

Table 1. *Semen profiles of the samples used in this study*

Concentration (10^6/ml)	Total count (10^6/ejaculate)	Motility (%)	Normal morphology (%)
106.34 ± 7.90	391.32 ± 30.05	58.05 ± 1.65	37.97 ± 1.44

Values = mean ± SE.

The slides were subsequently incubated with rabbit anti-mouse immunoglobulin (Z259; DAKO Ltd.), diluted 1:25 for 30 minutes, washed twice with Tris-buffered saline, and finally developed for 1 hour with an alkaline phosphatase–anti-alkaline phosphatase complex (D651; DAKO Ltd.) at a dilution of 1:50. Efficient labeling was then ensured by a repeat cycle of incubation with the alkaline phosphatase–anti-alkaline phosphatase complex. Alkaline phosphatase activity was detected by incubating the slides for 18 minutes with a substrate containing naphthol As-MX phosphate (0.5 mM), 2% dimethylformamide, 0.01 M levamisole, and 3.9 mM Fast Red TR in 0.1 M Tris buffer, pH 8.2. The slides were finally counterstained with hematoxylin and mounted in Apathy's aqueous mounting medium. At least 30 microscopic fields were counted, and from a knowledge of the volume equivalent of each high-powered field and the concentration of spermatozoa in the suspension, the number of leukocytes could be ascertained and expressed in relation to the volume of seminal fluid (per ml), as a function of sperm number (leukocytes/10^7 spermatozoa) or as a total leukocyte count for the ejaculate. Each analysis was carried out in duplicate and the mean value calculated; the minimal number of leukocytes that could be detected with this protocol was 0.02 × 10^6/ml.

Sperm Preparation

The spermatozoa were separated from the seminal plasma by discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient centrifugation using a two-step gradient comprising a 3-ml layer of 80% Percoll overlaid with 3 ml of 40% Percoll (Aitken and Clarkson, 1988). Isotonic Percoll was created by supplementing 10 ml of 10× concentrated medium 199 (Flow Laboratories, Irvine, Scotland) with 300 mg bovine serum albumin (BSA), 3 mg sodium pyruvate, and 0.37 ml of a sodium lactate syrup, and adding 90 ml of Percoll. This preparation was designated 100% Percoll (Lessley and Garner, 1983) and was subsequently diluted with HEPES-buffered Biggers Whitten Whittingham (BWW) medium (Biggers et al, 1971; Aitken and Clarkson, 1988). After a 20-minute period of centrifugation at 500 × *g*, the spermatozoa at the 40%:80% interface and at the base of the 80% fraction were collected separately, washed with a 5-ml volume of BWW medium and finally resuspended in BWW medium at a concentration of 20 × 10^6/ml.

Hamster Oocyte Penetration Test

The isolated spermatozoa were diluted 1:1 with the ionophore A23187, formulated as the free acid, to give final concentrations of 1.25 and 2.5 μM, respectively. In view of the reported vari-

Table 2. *Leukocyte and germ cell composition of the ejaculates analyzed in this study*

Cell type	Cells (10^4/10^7 spermatozoa)	Cells (10^4/ml)	Cells (10^4/ejaculate)
Total leukocytes	6.41 ± 2.41	39.19 ± 9.05	140.93 ± 35.31
B cells	0.46 ± 0.12	3.77 ± 0.98	15.51 ± 4.01
T cells	0.78 ± 0.18	6.06 ± 1.58	21.07 ± 5.54
Granulocytes	5.61 ± 1.64	39.69 ± 7.63	150.03 ± 30.04
Macrophages/ monocytes	0.26 ± 0.08	3.83 ± 2.01	13.3 ± 6.1
Germ cells	4.11 ± 0.66	43.49 ± 8.04	159.41 ± 29.91

Values = mean ± SE.

ation between donors in the optimal dose of ionophore to employ for the stimulation of sperm–oocyte fusion (Aitken et al, 1993b), each specimen was screened at both concentrations of A23187, and the highest result taken to represent the sample's potential for sperm–oocyte fusion. A23187 free acid was prepared as a 100 mM stock solution in dimethylsulfoxide and stored at −20°C. This stock solution was diluted to 1 mM with BWW medium and stored at 4°C until the day of experimentation, when it was diluted to the final working concentration. The spermatozoa were incubated with ionophore for 3 hours at 37°C in an atmosphere of 5% CO_2 in air and were then pelleted by centrifugation at 500 × *g*, resuspended in the same volume of fresh BWW medium, and distributed as 50-μl droplets under liquid paraffin. Zona-free hamster oocytes were prepared as described in the original publication of Yanagimachi et al (1976) and dispensed into the droplets at 5 oocytes/droplet and 15–20 oocytes/sample.

After a further 3 hours, the oocytes were recovered from the droplets, washed free of loosely adherent spermatozoa, compressed to a depth of about 30 μm under a 22 × 22 mm coverslip on a glass slide, and assessed for the presence of decondensing sperm heads with an attached or closely associated tail by phase contrast microscopy. The number of spermatozoa penetrating each egg was assessed and the results expressed as the mean number of spermatozoa penetrating each oocyte (total number of penetrations/total number of oocytes).

Acrosome Reaction

When time and material permitted, the acrosome reaction was assessed on the ionophore-treated sperm populations that were used for the zona-free hamster oocyte assay. At the end of the 3-hour incubation period with A23187, a 200-μl volume of the sperm suspension was removed, pelleted by centrifugation at 500 × *g* for 5 minutes, and resuspended in the same volume of fresh BWW medium prior to the assessment of sperm motility and acrosomal status. The protocol developed for assessing the acrosome reaction involved the use of a detection reagent targeting the acrosomal region of the sperm head, in conjunction with the hypoosmotic swelling test (Jeyendran et al, 1984) to monitor sperm viability (Aitken et al, 1993b). For this procedure, 50 μl of this sperm suspension was added to 500 μl of hypoosmotic swelling medium, comprising 7.35 g sodium citrate and 13.51 g fructose in 1 L of distilled water (Jeyendran et al, 1984), and incubated for 1 hour at 37°C. At the end of this period the spermatozoa were pelleted by centrifugation at 500 × *g* for 5 minutes and resuspended in 50 μl of ice-cold methanol. Ten μl of the fixed cells were subsequently pipetted onto the well of a four-spot Hendley slide (C. A. Hendley Ltd., Loughton, England) and allowed to dry. The wells were then overlaid with fluorescein-conjugated peanut lectin (*Arachis hypogaea*; Sigma Chemical Co., St. Louis, Missouri) at a concentration of 2 mg/ml and incubated for 15 minutes in the dark (Mortimer et al, 1987). The excess lectin was then gently removed by washing the slides with PBS and visualized using a fluorescence microscope in the presence of an anti-quenching agent (Citifluor, London, England). The spermatozoa were classified as non-acrosome-reacted if the acrosomal region of the sperm head exhibited a uniform, bright fluorescence and the sperm tails adopted the coiled configuration typical of viable cells. However, if the acrosomal region of such viable cells exhibited a punctate labeling pattern or restriction of the fluorescence to the equatorial segment of the sperm head, then they were classified as undergoing the acrosome reaction (Mortimer et al, 1987). Spermatozoa with straight tails were considered to be non-viable and were not included in the analysis (Aitken et al, 1993b).

Statistics

Frequency distributions for the data were prepared and, where necessary, transformations (log, log[1 + *x*], $\sqrt{}$) were used to normalize the data. Linear regression analysis was subsequently performed using the 'Statview' program (Abacus Concepts Inc., Berkeley, California) on an Apple Macintosh SE 30 computer.

Results

Leukocyte Composition

The composition of the seminal leukocyte populations analyzed in this study is presented in Table 2. In 120 samples, the number of CD45-positive leukocytes and CD45-negative precursor germ cells was determined, and in 91 of these samples, the leukocyte population was differentiated into B cells, T cells, monocytes/macrophages, and granulocytes (Table 2). In a great majority (97%) of specimens, leukocytes could be detected, and in 75/91 (82.4%) of these samples the predominant cell type was the granulocyte, the correlation between CD45-positive leukocytes and granulocytes being $r = 0.950$ ($P < 0.001$) on the raw data and $r = 0.890$ following log(1 + *x*) transformation (Fig. 1a). In 12 of these cases the concentration of granulocytes exceeded the WHO (1992) threshold for leukocytospermia of 1×10^6 cells/ml seminal fluid. The second most common species of leukocyte present in the human ejaculate was the T cell (Table 2). This cell type was present in 62% of samples examined and in four cases the concentration of T cells exceeded the number of granulocytes to become the predominant form of leukocyte. B cells could also be found in 43% of ejaculates, and in six cases (6.6%) they represented the major form of leu-

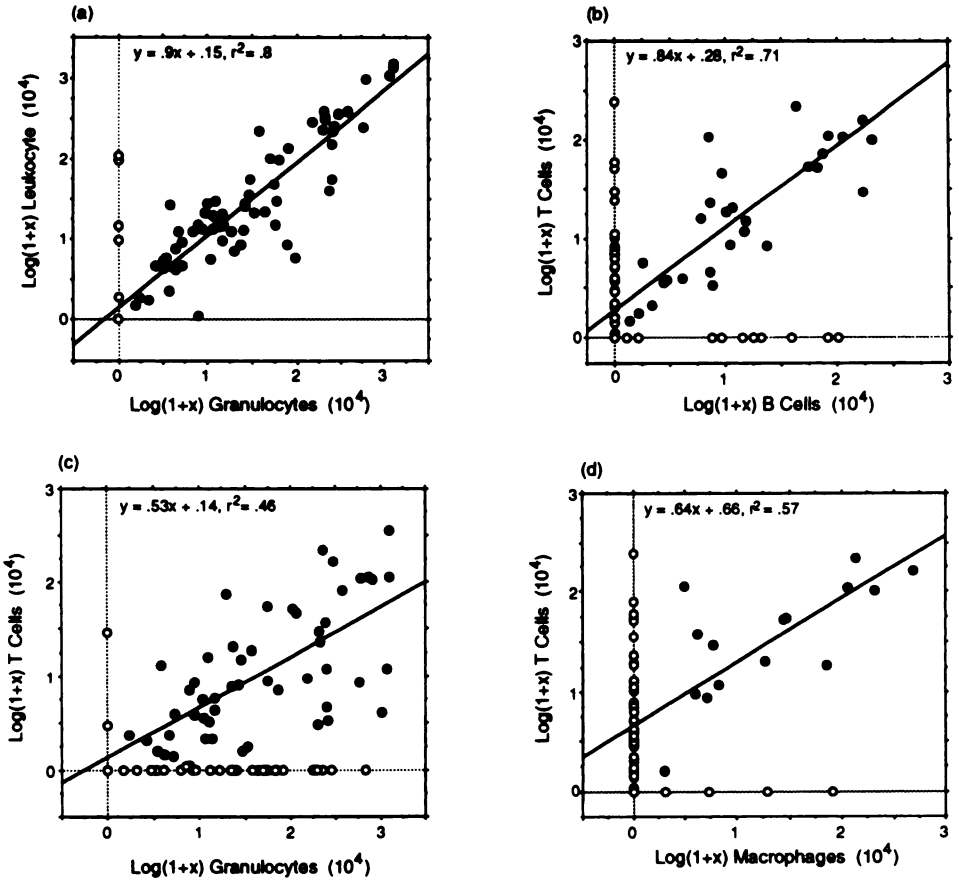

FIG. 1. Relationships between leukocyte subpopulations in the human ejaculate. (a) Granulocytes and CD45-positive leukocytes, (b) T and B cells, (c) T cells and granulocytes, and (d) T cells and macrophages. Note that for each panel, highly significant correlations were observed when both leukocyte species were present in an ejaculate (regression lines and closed symbols); samples containing only one type of leukocyte are indicated by open symbols. Data presented as log(1 + x) leukocytes × 10^4/ejaculate.

kocyte present in the ejaculate. However, even in cases where B or T cells predominated, the level of infiltration was low and never exceeded 1 × 10^6 cell/ml. Cells of the monocyte/macrophage lineage could also be detected in 21% of the samples examined, and in three (3.3%) cases constituted the predominant cell type, exceeding 1 × 10^6 cells/ml in one instance.

The extent to which the different leukocyte species infiltrated the human ejaculate was significantly correlated, particularly strong relationships being observed between the concentrations of monocytes/macrophages and B cells (r = 0.635), granulocytes and T cells (r = 0.529), and the two classes of lymphocyte (r = 0.491). However, analysis of the scattergram plots describing these data suggested that simple linear regression analysis was not adequate to describe the true relationship between the different classes of leukocyte.

Thus, plots of the raw data describing the relationship between the different classes of lymphocyte revealed that in those samples in which both classes of lymphocyte were present (Fig. 1b, closed symbols), their numbers were highly correlated, giving r values of 0.810 (/ejaculate) and 0.745 (/ml semen). Similarly, although the number of granulocytes in each ejaculate was significantly correlated with the number of B (r = 0.342; $P < 0.001$) and T cells (r = 0.529; $P < 0.0001$) when the entire data set was examined, if the analysis was confined to only those samples in which both types of leukocyte were present, then higher levels of correlation were observed, giving r values of 0.584 and 0.678, respectively (Fig. 1c). A similar picture emerged with respect to those samples exhibiting an infiltration of macrophages in association with other leukocyte species, in which particularly high correlations were observed between T cells (r = 0.754; $P < 0.001$; Fig. 1d), B cells (r = 0.676; $P < 0.001$), and the size of the macrophage population.

The Semen Profile

The presence of multiple leukocyte species in the human ejaculate was significantly associated with the presence of exfoliated germ cells. Overall, the presence of these cells was moderately but significantly correlated with the size of the various leukocyte subpopulations in the human ejaculate (Table 3). However, if the analysis was confined

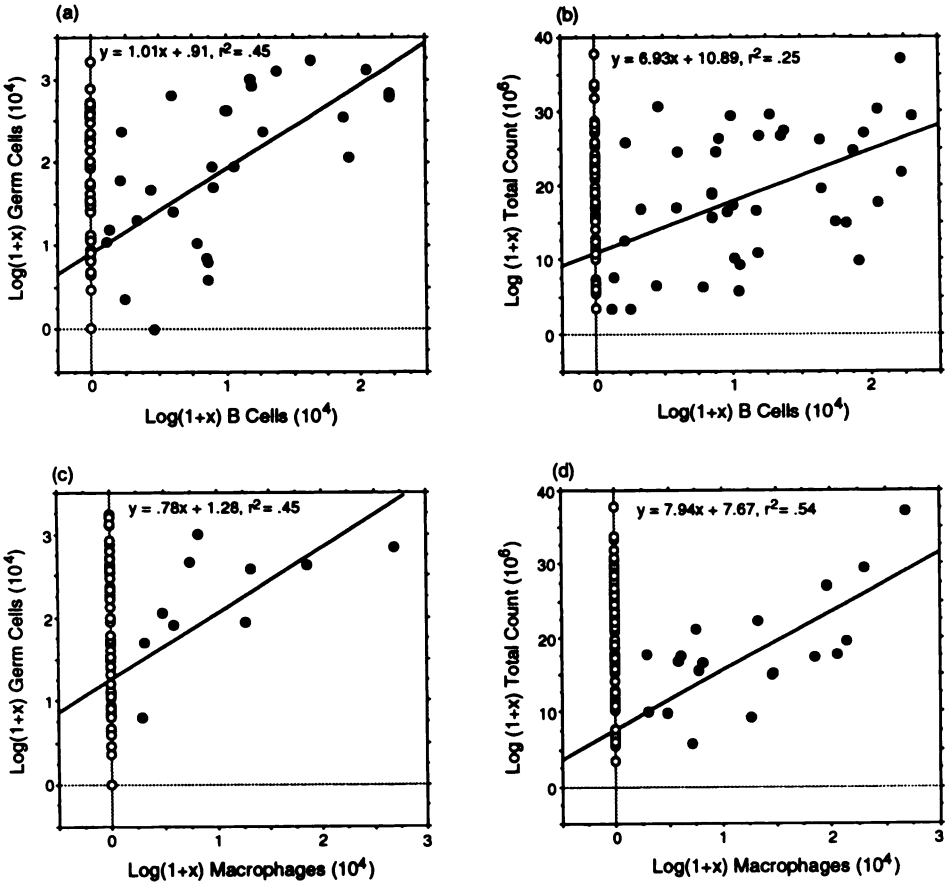

FIG. 2. Relationship between leukocytic infiltration and the numbers of precursor germ cells and spermatozoa in the ejaculate. **(a)** B cells and germ cells, **(b)** B cells and spermatozoa, **(c)** germ cells and macrophages, and **(d)** spermatozoa and macrophages. In those samples exhibiting evidence of leukocytic infiltration (regression lines and closed symbols), significant correlations with germ and sperm cell numbers were observed. Data are presented as the total count of each cell type in the ejaculate. Similar correlations were observed if these numbers were expressed as the concentration of each cell type per ml of seminal plasma.

to those samples in which leukocytic infiltration was observed, then these correlations were markedly improved, as illustrated in Figure 2. The most significant correlations were observed with B cells and monocytes/macrophages, both of which gave r values of 0.67 (Fig. 2a,c). The presence of multiple leukocyte species was also correlated with the sperm count, the most significant correlations being observed between sperm number and the concentrations of either B cells ($r = 0.500$; $P < 0.001$) or monocytes/macrophages ($r = 0.735$; $P < 0.001$; closed symbols in Fig. 2b,d). It should be emphasized that these correlations with sperm number were only observed in those samples

Table 3. *Correlation coefficients (r) describing the relationship between the concentration of different leukocyte subpopulations in the human ejaculate*

Cell type	Germ cells	B cells	T cells	Granulocytes	Macrophages/ monocytes
Total leukocytes	0.474***	0.408***	0.615***	0.833***	0.276*
B cells	0.347***	1	0.491***	0.342**	0.635***
T cells	0.082^{ns}	0.491***	1	0.529***	0.487***
Granulocytes	0.316**	0.342**	0.529***	1	0.337**
Macrophages/monocytes	0.257*	0.635***	0.487***	0.337**	1

*** $P < 0.001$.
** $P < 0.01$
* $P < 0.05$.
^{ns} Not significant.

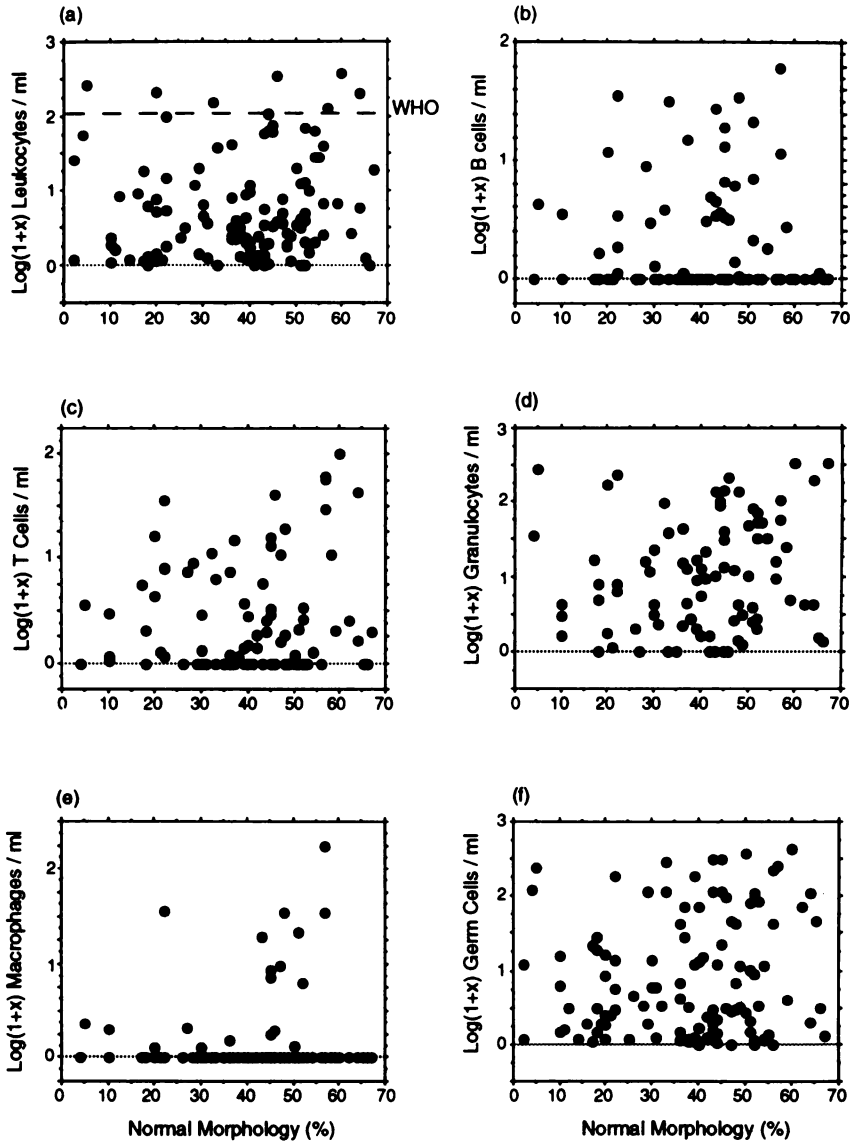

FIG. 3. Relationship between sperm morphology and the concentration of different leukocyte species and germ cells in the human ejaculate. Data presented as log(1 + *x*) leukocytes or germ cells × 10^6/ml; a similar lack of correlation is observed if the data are expressed in terms of the total number of each cell type in the ejaculate. '- - - WHO' represents the WHO (1992) criterion for leukocytospermia of 1 × 10^6 leukocytes/ml.

in which leukocytic infiltration was observed. If the entire study population was considered, then overall correlations with sperm concentration or total count were not observed. Moreover, within this study population, leukocytic infiltration, even to the point of leukocytospermia (>1 × 10^6 leukocytes/ml) had no influence on the morphology of the spermatozoa (Fig. 3) or their motility (data not shown), regardless of which attribute of movement was considered.

Sperm Function

Analysis of the functional competence of the spermatozoa revealed a similar picture, in that no correlation was observed between the concentration or total number of leukocytes in human semen and the rates of sperm–oocyte fusion or acrosome reaction subsequently observed with the Percoll-purified spermatozoa (Fig. 4).

In contrast, a profound influence of leukocyte contamination on the capacity of the washed sperm preparations for sperm–oocyte fusion was observed (Fig. 5a). In general, whenever leukocytes were present in these washed sperm preparations, sperm–oocyte fusion did not occur (Fig. 5a). This association was particularly true of the sperm populations isolated from the 40% Percoll fraction in which an association between leukocytic infiltration and failed fertilization was consistently observed (Fig. 5a). The only 40% Percoll fractions to show any capacity for sperm–oocyte fusion were those in which leukocyte con-

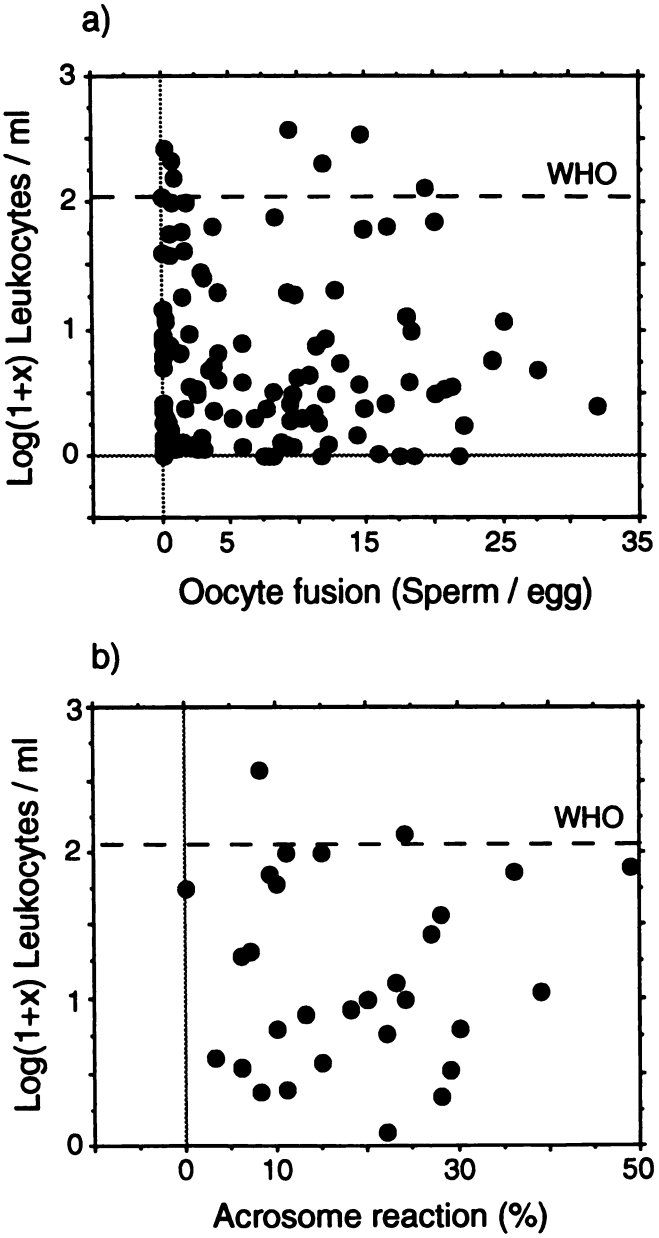

FIG. 4. Relationship between the presence of leukocytes in the human ejaculate and the subsequent ability of the spermatozoa to exhibit (**a**) sperm–oocyte fusion and (**b**) the acrosome reaction following their isolation on the 80% fraction of discontinous Percoll gradients and exposure to the ionophore A23187. Data are presented as log(1 + *x*) leukocytes × 10^6/ml; a similar lack of correlation was observed if the data were expressed in terms of the total number of each cell type in the ejaculate. '- - - WHO' represents the WHO (1992) criterion for leukocytospermia of 1 × 10^6 leukocytes/ml.

tamination was minimal or absent. Conversely, the 80% Percoll fractions generally exhibited high rates of sperm–oocyte fusion in the absence of leukocyte contamination. However, where leukocytes were observed, then sperm–oocyte fusion was usually impaired (Fig. 5a,b).

Contrary to the profound influence of contaminating leukocytes on sperm–oocyte fusion, the presence of these cells was not correlated with the capacity of the spermatozoa to undergo the acrosome reaction, following stimulation with A23187 (Fig. 5c).

In order to examine the mechanisms by which leukocyte contamination suppressed sperm–oocyte fusion, we undertook an analysis of the principal mechanism by which this component of sperm function is impaired—the generation of reactive oxygen species (Aitken and Clarkson, 1987; Aitken et al, 1989, 1991, 1992c; D'Agata et al, 1990; Iwasaki and Gagnon, 1992). Using chemiluminescence techniques to monitor reactive oxygen species generation (Aitken et al, 1992b), a clear relationship between high levels of this activity and the inhibition of sperm–oocyte fusion was observed (Fig. 5b). That this oxidative stress originated from the leukocyte population, rather than defective spermatozoa, was indicated by the excellent correlations observed between the degree of leukocyte contamination and the level of reactive oxygen species generation, giving correlation coefficients of 0.78 and 0.8 for the luminol and luminol plus peroxidase systems, respectively (Fig. 5d). Thus, in general, whenever leukocyte contamination was observed, high levels of reactive oxygen species were detected and sperm–oocyte fusion was impaired.

Discussion

Elucidating the nature, cause, and significance of leukocytic infiltration into the human ejaculate is of importance to our understanding of such issues as the transmission of retroviruses by seminal T cells (Anderson and Hill, 1987; Anderson, 1992), the role of B cells and macrophages in the mediation of autoimmune responses to sperm antigens (London et al, 1985; Wolff and Anderson, 1988), and the contribution of seminal granulocytes to the disruption of human sperm function through the creation of oxidative stress (Aitken and West, 1990; Aitken et al, 1992c; Kessopoulou et al, 1992). The results obtained in this study clearly indicate that leukocytic infiltration is a common feature of the human ejaculate and that the major cell type involved is the granulocyte. This is in agreement with El-Demiry et al (1986a,b), Wolff and Anderson (1988), and Tomlinson et al (1992), all of whom used monoclonal antibodies to identify the major leukocyte subpopulations. Reports that lymphocytes constitute the major cell type in the human ejaculate (Couture et al, 1976) were probably due to a failure to distinguish this leukocyte species from precursor germ cells. Moreover, the use of peroxidase activity as a marker for polymorphonuclear leukocytes, as in the study of Couture et al (1976), may have led to an underestimate of granulocyte number, because at least some of these cells appear to be in an activated state in human semen (Aitken and West,

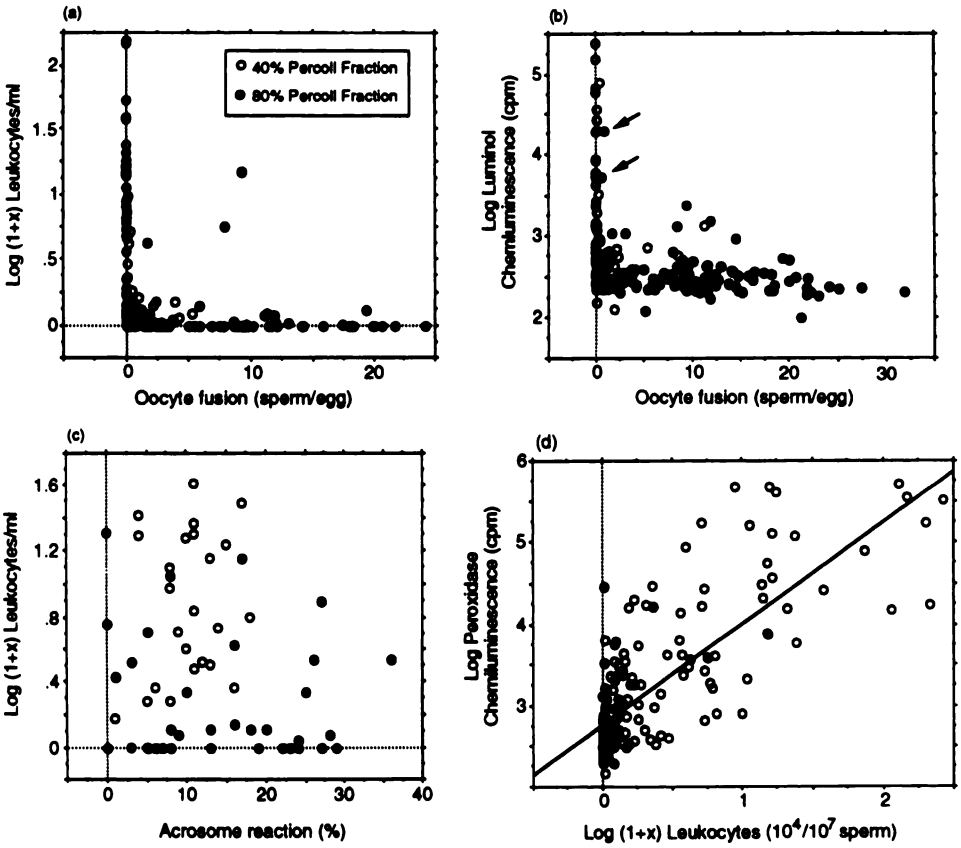

FIG. 5. Relationships among reactive oxygen species generation, leukocyte infiltration, and human sperm function. Leukocyte concentrations (log[1 + x] × 10^4/ml) were measured in the washed sperm populations containing 10^7 spermatozoa/ml isolated on the 40% (open symbols) and 80% (closed symbols) regions of discontinuous Percoll gradients. **(a)** leukocyte concentrations in the sperm preparations in relation to sperm–oocyte fusion, **(b)** sperm–oocyte fusion in relation to reactive oxygen species generation as measured by luminol-dependent chemiluminescence. Very similar data were obtained with luminol plus peroxidase-dependent chemiluminescence; arrows indicate two samples from the 80% Percoll fraction that contained low concentrations of leukocytes and in which the oxidative stress appeared to emanate from the spermatozoa rather than the leukocyte population. **(c)** Leukocyte concentrations in the washed sperm preparations in relation to the acrosome reaction, **(d)** leukocyte contamination in relation to reactive oxygen species generation as measured by luminol plus peroxidase-dependent chemiluminescence; the correlation coefficient was virtually identical if luminol-dependent chemiluminescence was plotted on the ordinate axis.

1990) and may have released their peroxidase-positive granules. In addition to granulocytes, this study also revealed the presence of other leukocyte species in the human ejaculate. Moreover, occasional cases were identified in which abnormally high concentrations of B cells, T cells, or monocytes/macrophages were present and exceeded the granulocyte concentration to become the predominant leukocyte species.

Surprisingly, only two previous studies have quantified the leukocyte subpopulations in the human ejaculate using monoclonal antibodies (Wolff and Anderson, 1988; Tomlinson et al, 1992), and the results obtained in these studies differed in many important respects. First of all, the general level of leukocytic infiltration was markedly different, giving a median CD45-positive count of over 1 × 10^6 per ejaculate (approximately 300,000/ml) in the patients analyzed by Wolff and Anderson (1988), compared with only 20,000/ml in the patient population examined by Tomlinson et al (1992). The median leukocyte count in our own study (91,000/ejaculate and 24,900/ml) is more in keeping with the Tomlinson et al (1992) data, as was the incidence of leukocytospermia (leukocyte concentration of more than 1 × 10^6/ml), which was 4.5% according to Tomlinson et al (1992) and 7.5% in the present study, compared with 23% in a recent analysis by Wolff et al (1990). The patient population studied by Wolff et al (1990) was also characterized by the presence of higher concentrations of monocytes/macrophages and lymphocytes than those examined by Tomlinson et al (1992) or ourselves. Such differences could easily arise as a result of different patterns of referral, and it is possible that the patient population examined by Wolff et al (1990) featured a higher incidence of genital tract infections, which would cause a disproportionate increase in the size of the leukocyte population as a consequence of local inflammatory reactions. Moreover, the poor semen quality observed by these authors in association with leukocytospermia may have been due to the impact of infection on secondary

sexual gland function (Gonzales et al, 1992) rather than a direct consequence of leukocyte infiltration per se. The present study clearly demonstrates that high concentrations of seminal leukocytes, beyond the WHO threshold of leukocytospermia ($>1 \times 10^6$/ml), are not detrimental and are fully compatible with a normal semen profile as well as normal sperm function, as measured by the hamster oocyte penetration test and bioassays of the acrosome reaction.

This apparently peaceful coexistence of leukocytes and spermatozoa in human semen must, in large part, be due to the antioxidant properties exhibited by seminal plasma (Jones et al, 1979), which protects the spermatozoa from the toxic oxygen metabolites generated by infiltrating granulocytes. However, in the absence of the protective properties of seminal plasma, leukocytes can be extremely damaging to sperm function. Thus, in the washed sperm preparations examined in this study, powerful relationships were observed among the presence of leukocytes, the generation of reactive oxygen species, and an impaired capacity for sperm–oocyte fusion. The ability of leukocyte-derived reactive oxygen species to disrupt sperm function is not only supported by the correlative data presented in this study but also by experimental evidence indicating that sperm–oocyte fusion can be disrupted by the addition of leukocytes to purified sperm suspensions (Maryama et al, 1985) and by chemically generated reactive oxygen species (Aitken et al, 1993a). Despite such data, the possibility that the associations among defective sperm function, oxidative stress, and leukocyte contamination are circumstantial, rather than causative, should also be considered. Thus, in the present study, it was predominantly the low-density Percoll preparations that were characterized by the presence of leukocytes, reactive oxygen species generation, and defective sperm–oocyte fusion. In such cell populations, morphological abnormalities in the spermatozoa might have been responsible for the loss of fertilizing potential, and the association of this phenomenon with leukocyte infiltration may have simply been a consequence of the fact that abnormal human spermatozoa and free radical-generating leukocytes exhibit similar densities and therefore locate in similar regions of the Percoll gradient. Notwithstanding such arguments, the existence of a causative element to these associations is strongly suggested by recent data revealing a significant increase in the rates of sperm–oocyte fusion as a result of the selective removal of leukocytes from washed suspensions of human spermatozoa using paramagnetic beads coated with an anti-CD45 monoclonal antibody (Aitken, unpublished observations).

The fact that the presence of leukocytes is associated with the suppression of sperm–oocyte fusion rather than the acrosome reaction reinforces recent data indicating that these two bioassays measure different aspects of sperm function and that acrosome reacted cells do not necessarily have the capacity to successfully fuse with the oocyte (Aitken et al, 1993b). These results have clear implications for the way in which washed human sperm populations are assessed for *in vitro* fertilization therapy and place emphasis on the need to develop simple methods (see Krausz et al, 1992) for assessing the extent to which such preparations are contaminated with leukocytes and then selectively removing these cells.

In addition to their therapeutic significance, the results obtained in this study may also have implications for our understanding of the etiology of leukocytic infiltration into the human ejaculate. Although genital tract infection is a probable causative factor in some cases, this is an extremely controversial area and it has been difficult to demonstrate a clear-cut relationship between the presence of pathogenic organisms in the ejaculate and the presence of leukocytes (Barratt et al, 1990). The present results suggest an alternative cause of leukocytic infiltration into the seminal compartment. Thus, in a large cohort of patients examined in this study there was a coordinated infiltration of multiple leukocyte species that positively correlated with the concentration of germ cells and, to a lesser extent, spermatozoa. The mechanisms underlying this set of associations are currently unknown, although the patients exhibiting this phenomenon were not distinguished by their age, any aspect of their clinical history, overt infections, or the functional competence of their spermatozoa. However, the existence of a cohort of patients in whom leukocytic infiltration is positively associated with the presence of germ cells and spermatozoa might explain the paradoxical observation made by El-Demiry et al (1986b) that leukocytes were more commonly encountered in fertile donors than infertile patients. It might also explain the positive association between sperm morphology and leukocytic infiltration reported by Tomlinson et al (1992), given that sperm morphology is frequently a reflection of sperm concentration ($r = 0.37$; $P < 0.001$ in this data set). Our current hypothesis is that in this particular cohort of patients the size and composition of the seminal leukocyte population is a consequence of semen quality, rather than a cause.

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