Enumeration of semen leucocytes by fluorescence in situ hybridisation technique

R A Conte, S Luke, R S Verma

Abstract

**Aim**—To determine whether the fluorescent in situ hybridisation technique (FISH) using a total human DNA genomic probe can be used to enumerate semen leucocytes.

**Methods**—Semen samples from five donors were subjected to a mild KCl solution. These samples were then biotin labelled under FISH conditions using a total human genomic DNA probe and the leucocyte counts were determined. To check the accuracy of the technique a monoclonal antibody against the common leucocyte antigen CD45 [KC56(T-200)] served as a control. An isotypic control for [KC56(T-200)], the immunoglobulin [MslgG1], served as a secondary control.

**Results**—Semen leucocytes stained by the FISH technique were easily detected because of their distinct bright yellow colour, while the sperm cells were red. The leucocyte counts ranged from 0·5 to 4·9 × 10⁶ per ml of semen. KC56(T-200) and its isotypic control MslgG1, which served as control for the FISH technique, accurately identified 94% and 97% of the semen leucocytes of a control donor, respectively.

**Conclusions**—The FISH technique using a total human DNA probe can accurately and effectively enumerate the overall leucocyte population in semen.

**Keywords:** Leucocytospermia, sperm, fluorescent in situ hybridisation.

Increased semen leucocyte counts have been correlated with inflammation and infection of male sex glands, asthenospermia, reduced sperm count, and infertility. The condition of leucocytospermia occurs when the leucocyte population exceeds 10⁶ per ml of semen. This condition may have characteristic infectious symptoms or a non-infectious origin and can be observed in fertile and infertile individuals. Activated leucocytes can produce cytotoxic effects which may reduce sperm motility, and impede the sperm's ability to penetrate hamster zona-free ova and human ova during in vitro fertilisation techniques.  

Semen macrophages have also played a role in phagocytosis of spermatozoa and have been associated with male infertility. Round cells in human semen are mainly leucocytes or immature germ cells. It has been suggested that conventional semen staining techniques are unreliable in completely differentiating immature germ cells from leucocytes. A need for a procedure that completely distinguishes semen leucocytes from immature germ cells in order to achieve an accurate germ cell count warranted new procedures, which have been assessed for their suitability. This investigation examines a process that uses a biotin labelled probe under fluorescent in situ hybridisation conditions, where the ability to detect leucocytes depends upon favourable selective differences between the respective leucocyte and germ cell designs for organisation of the hierarchical DNA folding arrangements and its nuclear density, coupled with the permeability and expansion potential of the nuclear membrane. A FITC conjugated monoclonal antibody against the common leucocyte antigen CD45 [KC56(T-200)] served as a control for the detection of semen leucocyte populations because it can detect all leucocyte types in semen.

**Methods**

After a three day period of sexual abstinence, semen specimens from five donors were collected in sterile containers. The semen volume was measured. Semen was allowed to liquefy for 30 minutes, mixed gently, and a small volume used for routine determination of motility and sperm number per ml. Motility was determined after placing a small drop on a slide and sealing it under a glass coverslip. Progressive motility was noted after one hour and three hours and measured on a range from 0 to 4. The sperm number per ml was calculated by diluting the semen using a white cell diluting pipette, with a standard dilution of 1:20, then counting the sperm in two leucocyte counting squares of a Neubauer chamber. KCl solution (0·075 M) was added to the remaining semen in a ratio of 1:3, mixed, and then incubated at 37°C for 20 minutes. Immediately afterwards, 0·25 ml of a methanol-glacial acetic acid fixative (3:1) was added and centrifuged at 1000 rpm for five minutes. The pellets were fixed twice. The remaining pellets were suspended with 2 ml of fixative per tube and dropped on precleaned slides. The slides were aged at room temperature for two days. The biotin labelled total human DNA genomic painting probe (Oncor) was performed as suggested by the manufacturer. The probes were biotin labelled, detected with fluorescein labelled avidin, and counterstained with propidium iodide.

The leucocyte number was calculated by separately counting the total number of sperm and leucocytes in 25 consecutive 250 × magnification fields on the FISH slides and routine control slides for each donor. The leucocyte number was divided by the sperm number, and this ratio was multiplied by the sperm number per ml to determine the leucocyte number per
Semen indices

<table>
<thead>
<tr>
<th>Donor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>4-5</td>
<td>2-5</td>
<td>2-5</td>
<td>4-0</td>
<td>3-0</td>
<td>2-5</td>
</tr>
<tr>
<td>Motility (0-4)</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sperm count (10⁶/ml)*</td>
<td>125</td>
<td>100</td>
<td>80</td>
<td>95</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Leucocyte/sperm ratio*</td>
<td>0-039</td>
<td>0-026</td>
<td>0-006</td>
<td>0-029</td>
<td>0-042</td>
<td>0-0094</td>
</tr>
<tr>
<td>Leucocytes (10⁶/ml semen)*</td>
<td>4-9</td>
<td>2-6</td>
<td>0-5</td>
<td>2-8</td>
<td>2-5</td>
<td>0-75</td>
</tr>
</tbody>
</table>

* Routine methods; † FISH method.

ml of semen. In order to check the specificity of the probe to detect only the semen leucocytes, a FITC labelled monoclonal antibody against the common leucocyte antigen CD45 [Coulter clone KC56 (T-200)] was used as a control for 100 cells in a staining sequence preceding the FISH technique (table). Observation of semen leucocyte populations at 250 x magnification was easily detected because they were displayed as bright yellow to green, while the sperm was red. Examples of the leucocyte population were observed at 1250 x (figure).

A sixth donor was used as a control to compare semen leucocytes stained by FISH with those stained by the immunocytochemicals [KC56(T-200)] and [MsIgG1]. The FISH results for semen leucocytes were 0-75 x 10⁶/ml; KC56(T-200) was 0-79 x 10⁶/ml and MsIgG1 was 0-77 x 10⁶/ml. In addition, a sequential control system showed that the FISH technique accurately determined 95 of 100 cells first determined by KC56(T-200) to be leucocytes (95%). The isotypic [MsIgG1] control system confirmed that 98% of the leucocytes detected by FISH technique were leucocytes.

Discussion

Presently, there is no analytical method to calculate sperm performance that will precisely determine male infertility, except in conditions where all sperm are aberrant. The majority of males who are tested for infertility either have low sperm count, asthenospermia, or abnormal morphology, and these often occur in combination. Leukocytospermia has been correlated with male infertility.9,31 Lymphokines and monokines produced by certain activated leucocytes have been responsible for cytotoxic effects on sperm under in vitro conditions and are believed to reduce sperm motility and have adverse effects on fertility.9,11 The duration of action and concentration of released cytokines, the sperm's proximity to the activated leucocytes in the male genital tract, and the quantity and type of leucocytes are all variables that affect the sperm.10,11 The epididymis is a site for sperm storage and passage where sperm can be susceptible to chemotactic and phagocytic effects9,12,33 that are most likely to be from granulocytes, monocytes/macrophages, and lymphocytes.1 If an increased round cell population in semen is composed of leucocytes this

Semen leucocytes revealed by a monoclonal antibody against the common leucocyte antigen CD45 (A,C) and sequentially detected by a total human DNA genomic painting probe (B,D) (1250 x).
Semleucocyte enumeration by FISH

may indicate a genital infection or there may be a subclinical disease; if the round cells are immature germ cells the cause is likely to be incomplete spermatogenesis. Routine semen staining procedures have not been completely reliable in distinguishing immature germ cells from leucocytes. Under specific conditions, the biotin labelled probe can selectively detect semen leucocytes, thus allowing them to be counted. A FITC conjugated monoclonal antibody against the common leucocyte antigen CD45, and its isotypic control (M1sg1G), which are able to detect T and B lymphocytes, monocytes, macrophages, granulocytes, thymocytes, and bone marrow cells, served as controls for the detection of semen leucocyte populations and confirmed the accuracy of the FISH technique. The reason why the semen leucocyte counts were slightly lower than with FISH with the immunocytochemical controls may be due to incomplete probe hybridisation with the target leucocyte DNA.

In order for a molecular probe to enter and hybridise with sperm DNA, it has to pass through its protective membrane. Lithium salts have been used successfully to expand the sperm membrane, allowing the probe to enter and hybridise.

If KCl is used, as described here, the sperm membrane will not expand sufficiently, preventing entrance and ultimately hybridisation of the probe. However, the leucocyte nuclear membrane will expand and the probe may enter and hybridise with the cell's DNA, allowing detection. The nuclear density of mammalian sperm DNA is six times more condensed than the DNA in mitotic genomes, and sperm chromatin organisation of protamines, which packages the DNA more compactly than any eukaryotic DNA, contains a vast network of disulphide bridges which must be reduced by strong agents to allow molecular probes to hybridise once they pass through the nuclear membrane. Also, the sperm nucleus plays an integral part in the design of this highly compact DNA organisation. These conditions have been considered obstacles in previous semen genomic DNA in situ hybridisation studies. The overall differences in the design of hierarchical DNA organisation and packaging of these two cell types apparently favoured leucocyte detection with the biotin labelled probe and allowed the enumeration of semen leucocytes.

We thank Michael W Lazar for the excellent photographs and JoLee M Pixley for her typing assistance.