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Formaldehyde-fixed semen is suitable and safer for leukocyte detection and DNA amplification*

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Infected semen is a principal mode of transmission of several human pathogens, including gonorrhea, human immunodeficiency virus (HIV), hepatitis B virus, chlamydia, and mycoplasms. To study semen infections, as well as protect andrology laboratory personnel from pathogen exposure, we have been interested in developing safer methods of routine semen analysis. Formaldehyde fixation of semen at the time of ejaculation is an attractive possibility because >2% formaldehyde solutions disinfect many pathogens (1), and detection of pathogen DNAs in formaldehyde-fixed pathology specimens is possible through polymerase chain reaction (PCR) amplification (2). Therefore, to take advantage of the disinfectant and stabilizing effects of formaldehyde fixation, we undertook studies of the feasibility of identifying cell types and pathogens in fixed semen samples.

Leukocyte-specific monoclonal antibodies (mAbs) have been reported (3, 4) to immunohistochemically distinguish semen leukocytes from other round cells such as immature forms of germ cells. Such a distinction is important to the design of effective treatment strategies for male reproductive tract pathologies. We report here that immunohistochemical identification of leukocytes is also possible in formaldehyde-fixed semen.

Although a number of reports indicate PCR will detect single copies of DNA sequences (2, 5) and thus provide a sensitive approach to the detection of genetic variations and pathogens in semen samples, at this time there are several obstacles that prohibit its routine use in andrology laboratories. Major drawbacks include the need for DNA purification, potentially infectious procedures not routine in andrology laboratories, and for radioactive or ligand-substituted DNA probes for hybridization to PCR products, also not routinely available in andrology laboratories. We report here the development of simplified PCR procedures suitable for formaldehyde-fixed semen. The procedures make it feasible to attempt to type genetic alleles in single fixed sperm (5) as well as detect pathogen DNAs (2).

MATERIALS AND METHODS

Immunohistochemistry of Formalin-Fixed Semen Cells

Semen samples were fixed in at least five volumes of buffered 3.0% to 3.7% formaldehyde or paraformaldehyde for a few hours (or up to 8 months). An aliquot of the cell suspension was centrifuged at 500 × g for 10 minutes, the supernatant solution removed, and the cell pellet washed twice by resuspension in phosphate-buffered saline (PBS) (10 mM sodium phosphate, pH 7.5, 154 mM NaCl). Aliquots

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of $\times 10^6$ cells in 5 μL were placed onto eight-spot slides, dried at 60°C, fixed in acetone, and immunostained with the leukocyte antibodies, DAKO-LCA, a mixture of two mAbs PD7/26/16 and 2B11 (Dako, Carpinteria, CA) and HLe-1 (Becton Dickinson, San Jose, CA), according to Zymed kit instructions (Histostain SP-kit; Zymed Laboratories, San Francisco, CA). Briefly, each spot was treated with 1% H_2O_2 for 5 minutes to block endogenous peroxidase activity, washed twice with PBS, and blocked with 10% nonimmune rabbit serum for 10 minutes, after which 50 μL drops of mAb (HLe-1, diluted 1:10 or LCA, diluted 1:25 in PBS) were applied for 60 minutes at 37°C in a humidified atmosphere. After rinsing with PBS, biotinylated rabbit anti-mouse immunoglobulin (diluted 1:200) was added for 10 minutes, followed by PBS rinses and treatment with streptavidin-conjugated horseradish peroxidase for 5 minutes. The substrate-chromogen complex (0.03% peroxide and aminoethylcarbazole) was added for 5 to 10 minutes. Slides were rinsed with distilled water and counterstained with hematoxylin to visualize nuclei and nonimmunoreactive cells.

Amplification of Sperm Genomic DNA Sequences in Formalin-Fixed Semen

Semen samples were divided into two parts, one of which was fixed with five volumes of formalin. Each part was washed and counted as for the immunostain procedure, and aliquots of known numbers of sperm were treated with 50% absolute ethanol for a minimum of 1 hour (up to 5 months), centrifuged in the cold at 10,000 rpm for 10 minutes, the supernatant decanted, and the pellet dried in a Speedvac (Savant, Farmingdale, NY). Dried pellets were resuspended in 10 μL of protease K buffer (10 mM Tris-HCl, pH = 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.5% NP40; 20 mM dithiothreitol 100 μg/mL protease K), digested overnight or for 2 days at 56°C, heated to 95°C for 10 minutes to inactivate the protease K and stored at −20°C before amplification. The initial PCR mixture (100 μL total including the digested sperm sample) contained 20 mM Tris-HCl, pH 8.8 at room temperature; 20 mM KCl; 2.5 mM $MgCL_2$; 10 μg/mL bovine serum albumin; 0.05% NP40; 3.5% ethylene glycol; 200 μM each dATP, dCTP, dGTP, and dTTP; 0.5 U *Taq* polymerase; and 10 pM of primers (low-density lipoprotein [LDL] receptor-specific primers derived from Genbank Accession No. L00347 and K02573, Intelligenetics, Inc., Mountain View, CA). Primer sequences were (5′ to 3′): AGTGCCAACCGCCTCACAG (LrM1) and CCTCTCACACCAGTTCACTC (LrM4). Cycling conditions were: two cycles at 98°C for 2 minutes, 55°C for 30 seconds, 72°C for 30 seconds followed by 48 cycles of 95°C for 1 minute, 55°C for 30 seconds, and 72°C for 30 seconds. Oil (a major source of crossover PCR contamination) was eliminated by performing the PCR under a heating blanket set at 95°C to minimize condensation on the lid of the reaction tube. All PCR tubes were handled with swatches of diethylaminoethane (DEAE) paper to further prevent crossover contamination (2). Reaction conditions for the second, heminested amplification were the same with the substitution of 1 U of *Taq* polymerase, 100 pM of primers LrM4 and LrM6 (TGGCTGGGTGAGGTTGTGGA, 5′ to 3′) and 1 μL of the first PCR products as sample.

Amplification of HIV DNA Sequences in Formaldehyde-Fixed Semen

Ejaculated semen from an HIV seropositive male was fixed in formalin at the site of collection and transported to the laboratory as a stable, disinfected cell suspension. Aliquots of semen cells were washed and immunostained, as described for Figure 1, and protease digested as described for Figure 2. The HIV PCRs employed three sets of primers to HIV *gag*, locus 1319-1659 (Genbank Accession No. K02007; Intelligenetics, Inc.) in a triple bracket/nested amplification strategy designed to amplify a single HIV sequence and shown to readily detect as few as five copies of control HIV provirus in the presence of $\times 10^5$ formaldehyde-fixed blood cells (2). The reaction conditions were the same as those described above with the substitution of HIV primers and variable amounts of polymerase. The primers used in each reaction were (5′ to 3′): TTATCAGAAGGAGCCACCCC and CCTTGTCTTATGTCCAGAATGC for the initial 341-base pair (bp) amplicon; AGTGGGGGGACATCAAGCAGCCATGCAAAT and CCTGCTATGTCACTTCCCCT for the intermediate 144-bp amplicon; and GAGACTATCAATGAGGAAGC and TGCTATGTCAGTTCCCCTTGGTTCTCT for the final 105-bp amplicon. The 341-bp sequence was amplified from 1 pM of each primer and 0.5 U of *Taq* polymerase (to limit nonspecific DNA synthesis) for two cycles of 98°C for 2 minutes; 55°C for 30 seconds; 72°C for 15 seconds; 18 cycles of 95°C for 1

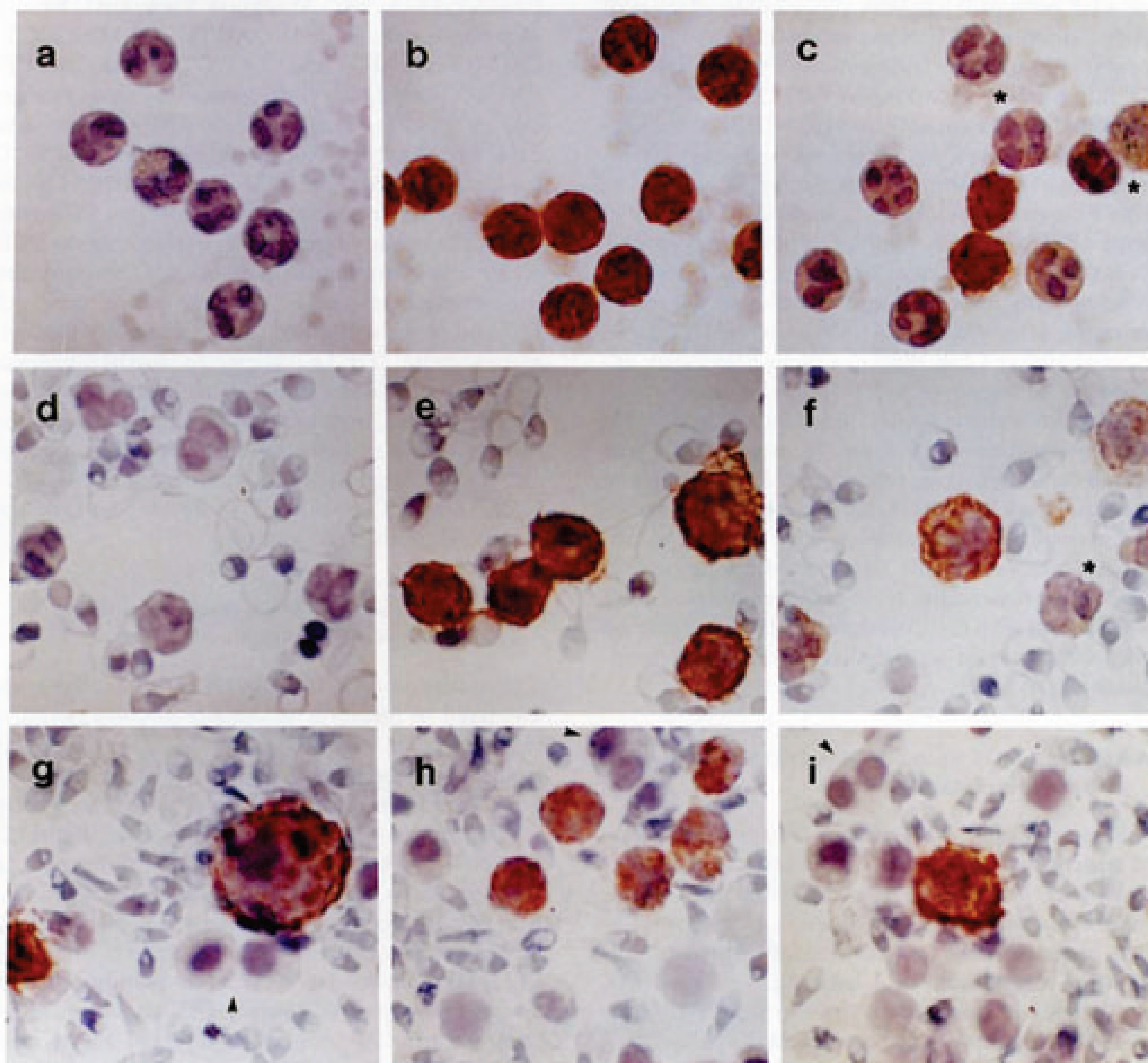

Figure 1 Immunostained formaldehyde-fixed leukocytes. Peripheral blood leukocytes and semen samples with leukocytospermia stained with hematoxylin (**A, D**) and immunostained with mAb HLe-1 (**B, E, G to I**) or LC (**C, F**), respectively. Immunopositive leukocytes are dark red-brown. Examples of nonimmunoreactive immature germ cells are designated by arrows (**G, H, I**). Examples of polymorphonuclear cells strongly immunoreactive with HLe-1 and weakly immunoreactive with LCA are designated by * (**C, F**). An example of a commonly seen, large semen cell immunoreactive with both mAbs is shown in **G.**

minute; 55°C for 30 seconds; 72°C for 15 seconds, plus a final 2-minute extension at 72°C after the 20 cycles. Ten microliters of the 100-μL final reaction products were transferred to a second, fresh PCR setup containing the same reaction components plus 100 pM of each primer for the 144-bp product and 2.5 U of *Taq* polymerase for two cycles of 98°C for 2 minutes; 55°C for 30 seconds; 72°C for 15 seconds; 38 cycles of 95°C for 1 minute; 55°C for 30 seconds; 72°C for 15 seconds, plus a final 2-minute extension at 72°C after the 40 cycles. For the final amplification, 2 μL of the 144-bp products were transferred to fresh reaction buffer containing 100 pM of the primers for the 105-bp product plus 2.5 U of enzyme for 40 cycles of the same strategy as the second amplification.

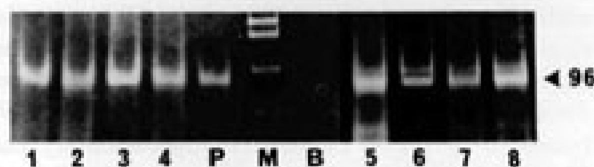

Figure 2 Amplification of LDL receptor DNA sequences in fresh and fixed sperm. Products from the nested amplification of LDL receptor were electrophoresed through 8.0% polyacrylamide and stained with eithidium bromide (3). Odd numbered lanes contain samples from fixed semen; even numbered lanes from nonfixed semen; *lanes 1* and *2*, $\times 10^6$ cells; *3* and *4*, $\times 10^7$ cells; *5* and *6*, $\times 10^5$ cells; *7* and *8*, $\times 10^4$ cells; P is 50 ng ($\times 10^4$ cell equivalents) of placenta DNA control; B is no DNA assay blank; M is Hae III digested phi X174 molecular size marker.

RESULTS

Figure 1 is an example of immunostained semen samples in comparison with peripheral blood cell controls (2). Under our conditions, the mAb, HLe-1, detected all formalin-fixed white blood cells (Fig. 1B), whereas LCA antibody detected principally lymphocytes and monocytes (Fig. 1C). Similar results were obtained with semen samples, such as the one in Figure 1D to F, that contained high concentrations of leukocytes: HLe-1 reacted with all leukocytes (Fig. 1E), and LCA bound principally lymphocytes and monocytes, reacting only faintly with granulocytes (Fig. 1F). In numerous semen samples, neither mAb reacted with fixed sperm or immature germ cells, as demonstrated by the semen cells immunostained with HLe-1 that contained a mixture of leukocytes and immature germ cells (Fig. 1G to I). Studies to more specifically identify the large semen cells (Fig. 1G) immunostained by HLe-1 (and LCA) are in progress.

To determine if the formaldehyde-fixation altered the immunohistochemistry of the semen cells detected, we conducted a comparison study of leukocyte concentrations in unfixed and fixed aliquots of semen samples obtained from men attending our andrology laboratory for routine semen analysis. As shown in Table 1, among semen samples with a wide range of cell concentrations, we found no significant differences in differential leukocyte counts between fresh and formalin-fixed preparations.

We began investigating the effect of formalin fixation on the PCR by amplifying LDL receptor sequences, previously studied in human sperm (5). Preliminary trials revealed that sufficient copies of a 254-bp amplicon were generated in 40 cycles from $\times 10^4$ nonfixed, protease-digested semen cells to be readily detectable by ethidium bromide (data not shown). However, the efficiency of the reaction was quenched by the fixation process, and we found that cell aliquots with $> \times 10^5$ sperm led to spurious, smeared DNA products, and those with $< \times 10^4$ sperm gave no detectable DNA products. Thus, it was necessary to modify the PCR protocol for formaldehyde-fixed sperm. The modifications included a second, nested amplification, the specificity of which was improved by decreasing the concentration of primers and polymerase in the first reaction, as described in Materials and Methods. These conditions made it possible to detect LDL receptor sequences in formaldehyde fixed semen cells ($\times 10^4$ to $\times 10^7$ per reaction) as a single, 96-bp DNA band in an ethidium stained gel at approximately the same level of sensitivity as unfixed cells (Fig. 2).

To determine the possibility of detecting pathogen DNAs in fixed semen, we carried out PCR amplification of HIV DNA sequences in semen from an HIV-infected man with hemophilia (Fig. 3). In this experiment, amplification of β-globin DNA sequences was used as the positive control for genomic cell DNA in the PCR mixtures. The sperm cell aliquot that contained $\times 10^7$ sperm and 8,700 leukocytes was positive for HIV DNA (Fig. 3, *lane 5*); the aliquot of one tenth this number of cells was negative. That the ethidium-stained bands were specific for the target sequence was verified by restriction enzyme digestion (data not shown) (2).

DISCUSSION

These findings define a stable, safe, rapid assay system for leukocytes and DNA sequences in

Table 1 Immunohistochemical Detection of Leukocytes in Semen

Patient	Sperm	Round	Cells: Fresh HLe+	Fresh LC+	Fixed HLe+	Fixed LC+
	$\times 10^6$/mL					
1	47	7.4	4.8	1.1	5.1	1.1
2	0.7	3.5	3.4	0.8	3.6	0.9
3	13	4.3	5.6	0.8	5.4	0.8
4	47	9.9	12.3	3.7	11.5	3.5
5	47	3.2	3.1	0.4	3.2	0.5
6	1.6	1.0	0.9	0.2	0.8	0.2
7	21	5.8	6.2	1.2	6.1	1.2
8	100	2.5	2.6	0.7	2.5	0.7
9	11	1.0	1.0	0.1	1.0	0.1
10	16	0.9	0.3	0.07	0.3	0.07

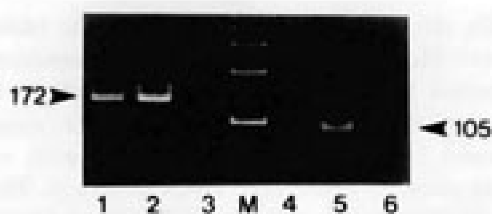

Figure 3 Amplification of HIV and β-globin gene DNAs in fixed semen cells. Two aliquots of semen that were formaldehyde fixed at the site of collection before transport to the laboratory were diluted in buffers and the cells collected for PCR analysis, as described in Materials and Methods. β-Globin DNA sequences were amplified as normal cell controls in a nested strategy employing the same PCR buffers and thermal cycling parameters described in Materials and Methods. Primer sequences for the first amplification (268-bp product) were (5' to 3'): CAACTTCATCCACGTTCACC and GAAGAGCCAAGGACAGGTAC; for the inner nested amplification (172-bp product): GTCATCACTTAGACCTCACC and TGGTGTCTGTTTGAGGTTGC. Initial PCR reactions for HIV sequences contained $\times 10^6$ and $\times 10^7$ sperm (870 and 8,700 leukocytes, respectively), *lanes 4* and *5*, respectively. Ten microliters of the reaction products were used as samples for the initial β-globin amplification, thus *lane 1* started from $\times 10^5$ sperm and *lane 2* from $\times 10^6$. *Lanes 3* and *6*: no DNA reaction blank controls. Final PCR products were electrophoresed through 8.0% polyacrylamide with a 123-bp ladder as a molecular size marker (M) and stained with eithidium bromide.

semen. Studies are in progress to determine if the formalin-fixed sperm have the same morphological characteristics as fresh cells that would make it possible to confine all aspects of a semen analysis except motility assessment to pathogen-inactivated cell suspensions. Our results show that, in fact, formaldehyde fixation significantly improves the morphology of round cells in semen relative to our standard semen smears, particularly the integrity of membranes, without reducing the sensitivity or specificity of immunostaining. The leukocyte antibody, HLe-1, reacted with essentially the same number of total leukocytes in both fresh and fixed semen samples with no detectable cross-reactivity with immature germ cells. However, in contrast to a previous report (4), but in agreement with an earlier study (6), we found that the leukocyte antibody, DAKO-LCA does not bind well to granulocytes, fresh or fixed, and is thus a relatively specific probe of lymphocytes/monocytes; there was no detectable cross-reactivity with immature germ cells. Thus, by immunostaining with HLe-1 and LCA, it is possible to distinguish and quantify all leukocytes versus lymphocytes/monocytes in formalin-fixed semen samples.

Because formalin fixation preserves the cells indefinitely, this approach is well suited to a clinical andrology laboratory setting in which samples could be preserved, pending a physician's request for a leukocyte analysis, or collected and preserved for analysis at sites remote from the laboratory. We found on average (Table 1) approximately one fourth of all HLe-1^+ leukocytes in semen were LCA^+ lymphocytes/monocytes (LC^+). Verifying such cell parameters in a larger study could lead to increased understanding of the etiology of leukocytospermia in general.

In addition, the ability to identify PCR-amplified cell and viral DNA sequences without the need for pathogen containment, DNA purification or labeled DNA probes paves the way for adaptation of these assays to clinical andrology laboratory settings. The addition of a third, nested PCR amplification of product is on the order of $\times 10^5$ more sensitive than the standard Southern blot/restriction digest approach to detecting PCR products. If it proves possible to also conduct gene allele studies with single, formalin-fixed sperm, this approach could be a valuable tool for retrospective analysis of elusive disorders such as idiopathic infertility.

By combining immunohistochemistry with PCR, it may be possible to quantify infected cells in semen by serial dilutions of cells in the initial PCR, thus providing rapid information about infection status or response to therapy. For example, leukocyte immunostaining revealed 870 LCA^+ cells per $\times 10^6$ sperm for the HIV^+ semen sample. The PCR assays containing $\times 10^7$ sperm (and thus 8,700 LCA^+ cells) were positive for HIV, and those containing $\times 10^6$ sperm were negative. If it can be proven that the PCR method was sufficiently sensitive to detect 1 infected cell and that only leukocytes and not sperm cells contain HIV DNA, at least 1 in 8,700 LCA^+ cells, but not as many as 1 in 870 LCA^+ would be HIV-infected in this semen sample. Further work is necessary to test this type of quantitative approach. Similar analyses may be possible with other semen pathogens, such as cytomegalovirus or ureaplasm.

SUMMARY

This pilot study indicates the use of formalin-fixation may offer a significant advance in the cytologic and pathological evaluation of semen. We are reporting this novel method to stimulate further assessment of this safer approach to semen analysis. The immunohistochemistry and PCR methods de-

scribed here may also prove useful in improving the safety of studies of pathogen-infected cells in other laboratory settings.

Key Words: Semen, formaldehyde-fixation, immunohistochemistry, human immunodeficiency virus, PCR.

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