Human semen induces interleukin 10 and 70 kDa heat shock protein gene transcription and inhibits interferon-γ messenger RNA production in peripheral blood mononuclear cells

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The influence of semen on immunity in sexually active women has been scarcely studied. The effect of human semen on production of messenger RNA (mRNA) for the anti-inflammatory TH2-related cytokine, interleukin-10 (IL-10), the 70 kDa heat shock protein (HSP70) and the pro-inflammatory TH1-related cytokine, interferon-γ (IFN-γ) was examined. Co-incubation of peripheral blood mononuclear cells (PBMC) from 10 women with a non-cytotoxic 1:50 dilution of semen lead to induction of IL-10 mRNA. Semen from each of seven different men tested induced IL-10 mRNA in PBMC. IL-10 protein was also released into the culture supernatant after PBMC–semen co-culture. Similarly, semen induced transcription of the HSP70 gene in PBMC obtained from 10 women. In contrast, semen did not induce IFN-γ mRNA in any of the female PBMC donors. Furthermore, semen markedly inhibited IFN-γ mRNA production without affecting cell viability in PBMC that were co-cultured with phytohaemagglutinin, a potent IFN-γ-inducing T-cell mitogen. Thus, human semen is both an inducer of an anti-inflammatory (TH2) immune response and an inhibitor of pro-inflammatory (TH1) cell-mediated immunity.

Key words: 70 kDa heat shock protein/interferon-γ/interleukin 10/immunosuppression/semen

Introduction

Mechanisms to inhibit immunity to spermatozoa within the female reproductive tract (James and Hargreave, 1984; Witkin, 1988) probably arose as a consequence of natural selection. Fertility was enhanced by development of mechanisms to foster sperm survival in the female after its deposition as well as to prevent immune sensitization to sperm-specific antigens.

Recently, Kelly et al. (1997a,b) and Jeremias et al. (1997) have described mechanisms of semen-induced immune suppression that may also facilitate the seminal transmission of microbial pathogens. Human semen was shown to induce the release of interleukin-10 (IL-10) from peripheral blood mononuclear cells (PBMC) while inhibiting interleukin-12 (IL-12) production (Kelly et al., 1997a). IL-10 is an anti-inflammatory cytokine and a potent inhibitor of cell-mediated immune responses as well as an inducer of humoral (TH2) immunity. IL-12 is an inducer of cell-mediated (TH1) immunity and a pro-inflammatory cytokine.

Under non-stressed conditions, the 70 kDa heat shock protein (hsp70) interacts transiently with nascent polypeptides to facilitate proper folding and promote protein translocation across intracellular membranes. During stress conditions, hsp70 synthesis is greatly enhanced and hsp70 forms a complex with proteins that fold unnaturally or denature, protecting these proteins from irreversible damage or targeting them for proteolytic cleavage (Kantengwa et al., 1991; Burel et al., 1992). HSP70 gene transcription also inhibits production of mRNA for the pro-inflammatory cytokines, interleukin-1 and tumour necrosis factor (Hall, 1994; Cahill et al., 1996). In addition, hsp70 facilitates the replication or induction of several viral pathogens including human immunodeficiency virus (HIV) (Stanley et al., 1990; Santoro, 1994). The ability of human semen to induce transcription of the gene coding for hsp70 in a human cervical cell line and in the endocervix following coitus was recently demonstrated (Jeremias et al., 1997).

In the present study we have examined the capacity of human semen to induce transcription in PBMC of the genes coding for IL-10, hsp70 as well as for the pro-inflammatory cytokine interferon-γ (IFN-γ). The results confirm and extend the concept that human semen both promotes anti-inflammatory TH2 immune responses and hsp70 production and inhibits pro-inflammatory TH1 responses and, thus, may facilitate pathogen transmission via coitus.

Materials and methods

Subjects

Female staff (n = 10) members of reproductive age were PBMC donors. Males of proven fertility were semen donors. All subjects were in good health at the time of sample collection and were negative for antisperm antibodies by the immunobead binding assay (Bronson et al., 1984). Semen samples were all within normal limits for sperm count, morphology, motility and visual absence of lymphoid cells.

Isolation of PBMC

Heparinized blood was diluted 1:1 in phosphate-buffered saline (PBS), underlayed with Ficoll–Hypaque lymphocyte separation...
medium and centrifuged at 1060 g for 15 min. The cell interface layer was washed three times with sterile PBS and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 25 mM HEPES, 1% L-glutamine, 20 µg/ml gentamicin and 10% heat inactivated autologous serum.

**PBMC culture conditions**

PBMC (10^6 PBMC/ml) were cultured in 6 wells for each combination for 20 h at 37°C in 5% CO₂ in complete medium supplemented with 10% heat inactivated autologous serum and either medium (as a negative control), phytohaemagglutinin (PHA; Gibco BRL Life Technologies, Grand island, NY, USA) 5 µg/ml as a positive control or a 1:50 dilution of whole semen. After the incubation an aliquot was saved from each cell culture and viability was performed by Trypan Blue exclusion. Cell viability was always >90%. The six replicate PBMC cultures were pooled and centrifuged at 3448 g for 5 min at room temperature and the supernatant saved and frozen at –80°C until cytokine analysis was performed.

**RNA isolation**

The pooled PBMC aliquots for each culture combination were lysed by resuspension in 0.2 ml Tris–HCl, pH 7.5 containing 0.15 M NaCl, 1.5 mM MgCl₂, 0.65% Nonidet P-40 detergent and 1 µl RNA-guard (Pharmacia, Piscataway, NJ, USA) and vortexing. Following centrifugation for 5 min at 3448 g the supernatant was removed and admixed with an equal volume of 7 M urea, 1% sodium dodecyl sulphate (SDS), 0.35 M NaCl, 10 mM EDTA, and 10 mM Tris–HCl. An equal volume of a 50:50:1 mixture of phenol:chloroform:isoamyl alcohol was added and the samples were vortexed and centrifuged at 12 752 g for 4 min at 4°C. The RNA underwent a double extraction procedure to ensure RNA purity after which the aqueous phase was removed, carefully avoiding any interface material, and two volumes of ice cold (~20°C) 100% ethanol plus 0.1 volume 3 M sodium acetate was added. The RNA was precipitated overnight at ~20°C, pelleted by centrifugation at 12 752 g at 4°C for 15 min and washed in ice-cold 70% ethanol to remove the sodium acetate. The RNA pellet was dried by desiccation for 20 min and diluted in diethyl pyrocarbonate-treated H₂O. Samples were stored at –80°C until reverse transcription–polymerase chain reaction (RT–PCR) were performed.

**Reverse transcription of cellular RNA to obtain complementary (c)DNA**

RNA was incubated for 15 min at room temperature in 20 mM Tris–HCl containing 50 mM KCl, 2.5 mM MgCl₂ and 1 IU of DNase I (Gibco BRL Life Technologies). The reaction was terminated by the addition of 2 mM EDTA and heating for 10 min at 65°C. Reverse transcription of RNA to cDNA was performed using 600 IU murine Maloney leukaemia virus (MMLV) reverse transcriptase (Gibco BRL Life Technologies) in 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl₂, 100 µg/ml bovine serum albumin (BSA), 0.5 mM each dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, USA), 1330 IU/ml RNAsin ribonuclease inhibitor (Promega), and 2 µg oligo dT₁₅ (Promega). After a 60 min incubation at 37°C, the samples were heated to 95°C for 5 min, cooled to 4°C and stored at –80°C until PCR was performed. One aliquot of cDNA was amplified for each experiment and the PCR products detected in duplicate by enzyme-linked immunosorbent assay (ELISA) (see below). The average of two ELISA values is shown (see results).

**Quantitative PCR for cytokine and HSP70 mRNA expression**

Aliquots of cDNA were combined with reaction buffer (10 mM Tris–HCl, 1.5 mM MgCl₂, 50 mM KCl, 200 µM dATP, dCTP and dGTP, 190 µM dTTP and 10 µM digoxigenin-11-2’-deoxyuridine-5’-triphosphate (dig-11 dUTP; Boehringer Mannheim, Indianapolis, IN, USA) and 15 pmol of oligonucleotide primers specific for IL-10, 12 pmol primers specific for IFN-γ, or 7.5 pmol primers specific for hsp70 (StressGen, Victoria, BC, Canada) (Table I) and 1.25 IU Taq DNA polymerase (Promega) in a total volume of 50 µl. cDNA samples were subjected to 30 cycles of varying temperatures: 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min for IL-10 or IFN-γ. This was followed by an extension cycle of 7 min at 72°C. The hsp70 amplification conditions were identical to β-actin and are given below. The generation of a 494 bp product for IFN-γ, a 360 bp product for hsp70, and a 352 bp product for IL-10 was confirmed by gel electrophoresis and ethidium bromide staining.

As an internal control each sample was examined for the presence of β-actin cDNA. cDNA samples were amplified, using the same reagent concentrations as above, in the presence of 6 pmol of human β-actin specific primers. The samples were subjected to one cycle at 95°C for 3 min, 48°C for 30 s, and 72°C for 1 min 30 s followed by 28 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 1 min 30 s, followed by a 5 min extension at 72°C.

By performing these quantitative PCRs as described above for no more than 30 cycles, the PCR products increased linearly with time and, therefore, the amount of PCR product was in direct proportion to the amount of template RNA. In our experience, PCR for 30 cycles is consistently in the linear range for amplicon production. Known concentrations of DNA (standard curve) were amplified in parallel to the test samples and used in the calibration of the ELISA detection assay.

As a positive control RNA was extracted and cDNA synthesized from PBMC from healthy women incubated in RPMI medium containing 10% heat inactivated autologous serum and 5 µg/ml PHA for 20 h. Negative controls (no cDNA) was amplified in parallel with test samples for all assays.

**ELISA detection of PCR products**

To increase sensitivity and specificity of mRNA detection all amplified PCR products were detected by ELISA following hybridization with a biotinylated oligonucleotide probe (Table I). Duplicate aliquots (10 µl) of digoxigenin amplified PCR products were denatured at 25°C for 10 min and hybridized with 7.5 pmol/ml of a biotinylated probe. Digoxigenin-labelled biotin PCR complexes were incubated for 3 h at the appropriate temperatures (Table I) in wells coated with streptavidin, and the bound product was detected using peroxidase conjugated anti-digoxigenin antibody (Boehringer Mannheim, Mannheim, Germany) and the colourimetric substrate ABTS. Wells containing no cDNA, and negative PCR product were used as negative ELISA controls.

The presence of β-actin was confirmed in all cDNA samples from the PBMC cultures. All cytokine and hsp70 PCR data were normalized for β-actin concentration.

**Cytokine analysis of PBMC cell culture supernatants**

All PBMC cell culture supernatants were analysed in duplicate for IL-10, and IFN-γ by commercial ELISA kits (Biosource International, Camarillo, CA, USA). Cytokine protein concentration was calculated from ELISA controls.

The average of two ELISA kits have the following sensitivities: IL-10, 0.2 pg/ml, and IFN-γ<4 pg/ml.

**Results**

**Induction of IL-10 mRNA in PBMC by semen**

IL-10 gene transcription was induced in PBMC from each of 10 women when incubated with a non-cytotoxic concentration...
Table 1. Primers for reverse transcription–polymerase chain reaction and biotinylated probes and hybridization temperatures for enzyme-linked immunosorbent assay (ELISA)

<table>
<thead>
<tr>
<th>Target cDNA</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Biotinylated probe</th>
<th>Hybridization temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AATCTGGCACACACCT</td>
<td>CGTCTACATCCCTGCTT</td>
<td>TCGTGCGTGACA</td>
<td>47°C</td>
</tr>
<tr>
<td>IL-10</td>
<td>TGCCCAAGGCTGCTGCTG</td>
<td>CGGCTCATTCCGCCG</td>
<td>CAGGACATCAAAGA</td>
<td>47°C</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ATGGAATATAACAGT</td>
<td>GATGCTCTTCGACCCTT</td>
<td>CAGGACATCAAAGA</td>
<td>47°C</td>
</tr>
<tr>
<td>hsp70</td>
<td>TGGTCTGGTTTCCAGCC</td>
<td>GGGCTGTCTCCGTCG</td>
<td>CAGGACATCAAAGA</td>
<td>51.1°C</td>
</tr>
</tbody>
</table>

IL-10 = interleukin-10; IFN-γ = interferon-γ; hsp70 = 70 kDa heat shock protein.
The primers are written 5’ to 3’. The probes for the ELISA detection are all biotin labelled at the 5’ end.

Figure 1. Induction of interleukin-10 (IL-10) mRNA by semen in peripheral blood mononuclear cells (PBMC). PBMC from 10 female donors were incubated with whole semen from a fertile male (□), phytohaemagglutinin (PHA; □) or medium (■). The presence of mRNA for IL-10 determined by polymerase chain reaction/enzymelinked immunosorbent assay. OD = optical density.

Figure 2. Induction of interleukin-10 (IL-10) mRNA in peripheral blood mononuclear cells (PBMC) by different semen samples. PBMC from a single female donor was incubated with a 1:50 dilution of whole semen from seven different fertile men (□), phytohaemagglutinin (PHA; □) or medium (■) and IL-10 mRNA levels were measured as described in Figure 1. OD = optical density.

Induction of HSP70 gene transcription by human semen
Human semen also induced transcription of the HSP70 gene in PBMC from each of 10 women (Figure 7). In eight out of the 10 cases, the amount of mRNA induced by semen was greater than that induced by PHA.

Discussion
T lymphocyte-derived immunity is divided into two distinct responses, TH1 responses, associated with production of IFN-γ and IL-12, induce cell-mediated immune functions and pro-inflammatory activity, TH2 responses, associated with IL-10 and interleukin 4 production, induce humoral immunity and are anti-inflammatory (Seder and Paul, 1994). The results of the present investigation, when combined with the previous report by Kelly et al. (1997), demonstrate that human semen induces IL-10 gene transcription and protein synthesis in PBMC while inhibiting IL-12 and IFN-γ production. Thus, human semen stimulates the release of a potent TH2 anti-inflammatory cytokine while blocking synthesis of two TH1 pro-inflammatory cytokines. Since TH1 responses comprise the primary immune defense against viruses and other pathogens,
mRNA induction by human semen

Figure 3. Kinetics of interleukin-10 (IL-10) release into the culture supernatant after peripheral blood mononuclear cells (PBMC)–semen co-culture. PBMC from a female donor was incubated with a 1:50 dilution of whole semen (□–□) or phytohaemagglutinin (PHA; ●●●). Aliquots were removed at timed intervals and assayed in duplicate for IL-10 by enzyme-linked immunosorbent assay.

Figure 4. Induction of interferon-γ (IFN-γ) mRNA in peripheral blood mononuclear cells (PBMC) by semen. PBMC from 10 women were incubated with a 1:50 dilution of whole semen, PHA or medium for 20 h, and assayed for IFN-γ specific mRNA by reverse transcription–polymerase chain reaction/enzyme linked-immunosorbent assay.

Figure 5. Induction of interferon-γ (IFN-γ) mRNA by semen from different men. Peripheral blood mononuclear cells (PBMC) from a single female donor was incubated with a 1:50 dilution of semen from seven different men, phytohaemagglutinin (PHA) or medium and IFN-γ-specific mRNA determined by reverse transcription–polymerase chain reaction/enzyme linked-immunosorbent assay.

Figure 6. Effect of semen on phytohaemagglutinin (PHA)-induced interferon-γ (IFN-γ) mRNA production. Peripheral blood mononuclear cells (PBMC) were incubated in medium (●), PHA (■), PHA plus a 1:50 dilution of semen (●), or semen alone (□) for 20 h at 37°C. IFN-γ mRNA levels were measured by reverse transcription–polymerase chain reaction.

Figure 7. Induction of HSP70 mRNA in peripheral blood mononuclear cells (PBMC) by semen. PBMC from 10 different women were incubated with a 1:50 dilution of whole semen from the same male donor. After 20 h at 37°C, the presence of HSP70-specific mRNA was measured by reverse transcription–polymerase chain reaction/enzyme linked-immunosorbent assay.

Semen-induced abrogation of this activity would appear to increase susceptibility to infection by these microorganisms. The proliferation of Candida albicans in the human vagina is inhibited by cell-mediated immunity (Witkin, 1987) and development of symptomatic vulvo-vaginal candidiasis has been associated with frequent sexual intercourse (Spinillo et al., 1993; Geiger et al., 1995). This further emphasizes the ability of the human male ejaculate to promote those infections in the female genital tract that are normally controlled by cell-mediated immune mechanisms.

The present report, plus a previous communication (Jeremias et al., 1997), illustrate that semen also induces hsp70 mRNA production in both endocervical cells and PBMC. Hsp70 plays an important role in viral gene activation and replication and assembly of viral proteins. The cellular heat shock element and the enhancer region of cytomegalovirus (Geelen et al., 1987) and HIV (Stanley et al., 1990) have similar DNA sequences; hsp70 gene activation and viral genome activation may occur simultaneously. In macrophages infected with vaccinia virus, hsp70 concentrations are maximal at the peak
of viral gene expression and hsp70 is associated with viral proteins (Jindal and Young, 1992). Similarly, in adenovirus (Niewiarowska et al., 1992), poliovirus (Macejak and Sarnow, 1992) and Epstein–Barr virus (Mannick et al., 1995) infections, hsp70 is involved in viral protein folding and morphogenesis. Reactivation of latent herpes simplex virus (Moriya et al., 1994) and HIV (Stanley et al., 1990) has been detected following hsp70 induction by transient hyperthermia. Thus, exposure to semen may induce viral gene expression and/or replication in female genital tract cells that are latently infected and facilitate male to female sexual transmission of viral pathogens. Initiation of hsp70 gene transcription also leads to a decrease in pro-inflammatory cytokine production (Hall, 1994; Cahill et al., 1996).

Prostaglandins, especially E2 (PGE2), are present in seminal fluid in uniquely high concentrations compared to other body fluids and may be responsible for IL-10 induction and IL-12 and IFN-γ inhibition (Kelly et al., 1997a,b). PGE2 is known to inhibit IFN-γ expression and suppress PBMC proliferation. The decrease in pro-inflammatory cytokine production in mononuclear phagocytes following PGE2 exposure is inversely proportional to the increase in IL-10 production (Strassmann et al., 1994), suggesting that it may be the ability of PGE2 to trigger IL-10 synthesis that is responsible for this effect.

The inducer(s) of hsp70 transcription in semen remains unidentified. Any one of the multitude of cytotoxic or proteolytic components of this secretion (James and Hargreave, 1984) might be expected to induce a stress response in somatic cells. Recognition that human semen activates anti-inflammatory immunity and hsp70 expression enhances our understanding of the influence of the ejaculate on sexual transmission of microbial pathogens. Continued analysis of the molecular mechanisms responsible for semen-induced immune suppression will lead to novel strategies to enhance resistance to genital tract infections. Such studies are now in progress.

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References

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