Effects of urinary gonadotrophin preparations on human in-vitro immune function

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Urinary gonadotrophins have been used since 1962 in the treatment of ovulation failure (Lunenfeld et al., 1962) and also, in the past 15 years, for stimulating multiple follicular development prior to oocyte collection for assisted reproduction techniques. Despite the elimination of some other urinary proteins during the extraction of HMG, FSH and LH still account for approximately 5% of the total protein content of currently available HMG preparations (Loumaye et al., 1996). The specific activity of the HMG products does not usually exceed 150 IU FSH/mg protein (Giudice et al., 1994). Although, during stimulation of multiple follicular development for in-vitro fertilization (IVF) with conventional gonadotrophin preparations, approximately 15 mg or more of protein is administered, only about 0.2 mg of this is FSH or LH. The problem is that the extraneous proteins can cause unpredictable side effects that relate not only to a possible interference with the therapeutic action of the HMG product, but also perhaps to local reactions, pain and hypersensitivity reactions (Editorial, 1992; Harika et al., 1994; Odink et al., 1995; Redfearn et al., 1995; Albano et al., 1996).

The relevance of purity in the risk of allergic reactions has been confirmed, at least in experimental animals, by comparing the frequency and severity of allergic reactions induced by administration of different traditional human FSH and HMG preparations with those induced by a highly purified preparation. Severe allergic reactions (anaphylactic shock) occurred in 20% and 7% of guinea-pigs receiving highly purified FSH compared with the other non-highly purified preparations. Significantly lower IgE titres were similarly induced by highly purified FSH compared with the other preparations (Biffoni et al., 1994). In addition to immediate-type hypersensitivity reactions, contaminants might also provoke non-immediate immune-mediated side effects through T cell-mediated mechanisms triggered by antigenic stimulation or by pro-inflammatory mediators present in such preparations. To analyse the effects of urinary gonadotrophins on human immune functions, a panel of tests exploring their effects on T cell and monocyte function has been performed and the concentrations of selected cytokines, known to modify immune responses, were measured by immunoassay. In order to differentiate the potential effects induced by the gonadotrophins per se from the effects of other proteins contained in the preparations, the studies were also performed with a >95% pure urinary FSH preparation, a recombinant human (r-h) FSH preparation (>97% pure) and a r-hLH. The tests employed were: phytohaemagglutinin (PHA)-induced proliferation and mixed lymphocyte reaction (MLR) with human peripheral blood mononuclear cells (PBMC), IL-2 production from CD4+

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Introduction

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are heterodimeric glycoproteins that are secreted by the anterior hypophysis and which regulate gonadal function. Both hormones are excreted in the urine of post-menopausal women in biologically active forms and in high concentrations. Extraction of the hormones from the urine of such women provides a preparation referred to as human menopausal gonadotrophin (HMG).
Jurkat cells (a human T cell line that can be induced to produce IL-2), and IL-1 production from PBMC and the THP-1 monocytic cell line.

Materials and methods

Study drugs

The following preparations were tested in the human lymphoproliferation assays (PHA and MLR), as indicated in the results. (i) HMG extracted from the urine of post-menopausal women: five batches of Humegon® (Organon, Oss, The Netherlands) and five batches of Pergonal® (Ares-Serono, Geneva, Switzerland); (ii) human menopausal urinary FSH: three batches of Metrodin® (Ares-Serono); (iii) highly purified human menopausal urinary FSH: two batches of Metropin HP® (Ares-Serono); (iv) r-hFSH: four formulated and one pre-formulation batches of Gonalf F® (Ares-Serono); and (v) r-hLH: two pre-formulation batches of LHadi® (Ares-Serono).

Additional preparations were tested in the other assays and for their cytokine content.

Immunosassays

The following enzyme-linked immunosorbent assay (ELISA) kits were used to measure human cytokine concentration (quantitation limit is shown in brackets): IL-1α (3.9 pg/ml), IL-1β (3.9 pg/ml), IL-2 (31.3 pg/ml), IL-4 and tumour necrosis factor (TNF) α (15.7 pg/ml and TNFβ (31.3 pg/ml), all from Amersham (Milan, Italy), and interferon (IFN) γ (15 pg/ml) from CLB (Amsterdam, The Netherlands).

Cells

Human buffy coats from healthy blood donors were used as the source of PBMC and obtained from the Italian Red Cross (CRI, Rome, Italy). Blood samples had been previously checked at CRI for the presence of markers of infection arising from the HIV, hepatitis B and hepatitis C viruses and were used only if negative. PBMC from the same five different subjects were used to analyse the effect of the FSH-containing preparations (a single batch of each). Five different batches of Pergonal and Humegon were tested on PBMC from three subjects to analyse the batch to batch consistency of the observed effects.

The human B-lymphoblastoid cell line CESS-B was used in the MLR assay as the source of stimulating cells.

The THP-1 and Jurkat cell lines were purchased from ATCC (Rockville, MD, USA) as frozen ampoules and established in long-term culture, according to the instructions of the supplier.

Preparation of human PBMC

Buffy coats were diluted 1:4 with phosphate-buffered saline (PBS) (Biochrom KG, Berlin, Germany). This cell suspension was then carefully layered onto a Ficoll Hypaque separating solution (Biochrom KG), and centrifuged for 30 min at 900 × g, at 18–20°C. The mononuclear cell layer at the gradient interface was then harvested and washed by centrifugation for 10 min at 400 × g at 18–20°C, with an excess of complete medium composed of RPMI-1640 (Biochrom KG) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) or human AB serum (Sigma, St Louis, MO, USA), 2 mM l-glutamine (Bio-Whittaker, Verviers, Belgium) and 5 µg/ml gentamicin sulphate (Sigma). The washing procedure was repeated twice, and the PBMC were then resuspended in complete medium.

Spontaneous and PHA-induced proliferation

For the proliferation assay, 10^5 PBMC in 100 µl complete medium were dispensed into each well of a 96-well plate. Three wells were prepared for each experimental test. Cells with no mitogen were included as a background proliferation control and to evaluate the effects of gonadotrophin preparations on the spontaneous in-vitro proliferation of PBMC.

PBMC from different individuals were stimulated with PHA HA16 (Murex Diagnostics, Dartford, UK), at different concentrations, or left with no stimulus in the presence of different concentrations of the test substances. After a 3-day incubation period (37°C, 5% CO2), cells were pulsed with 1 µCi/well [3H]-thymidine (NEN, Cinsello Balsamo, MI, Italy). After an overnight incubation period, the cells were harvested and the [3H]-thymidine incorporated into each sample was quantified in a liquid scintillation counter.

The results are presented as the stimulation index (SI), defined as the ratio between the mean counts of the sample and the relevant control.

MLR

CESS-B cells (stimulating cells) and a sample of PBMC were incubated for 30 min at 37°C with 25 µg/ml mitomycin C (Sigma), to prevent their proliferation in the MLR assay. Residual mitomycin C was then removed by washing the cells three times in complete medium. Responder cells (100 µl of a PBMC suspension of 10^6 cells/ml in medium containing 10% human AB serum) were dispensed into each well of the assay plate (three replicates for each experimental condition). Mitomycin C-treated stimulator cells (50 µl at 2×10^6/ml) were added to the appropriate wells. Test or control substances were also then added (50 µl) to the respective wells. Plates were incubated for 5 days in a 37°C, 5% CO2 humidified incubator. After this period, 1 µCi of [3H]-thymidine was added to each well of the plate. After an 18-h incubation period, the cells were harvested and [3H]-thymidine incorporation evaluated by liquid scintillation counting. The results are presented as SI.

IL-1 induction

Human PBMC were isolated by Ficoll Hypaque centrifugation from different donors; THP-1 monocytic cells were used in the log phase of growth in culture.

PBMC and THP-1 were washed and resuspended in RPMI-1640 medium, added together with 10% FBS at the working cell concentration. The induction of PBMC was carried out in 48-well plates, with a final concentration of 5×10^5 cells/500 µl medium per well. The experiments with THP-1 were performed in 96-well plates with a final cell concentration of 1×10^5/250 µl medium per well. The test drugs were added to the wells, alone or in combination with lipopolysaccharide (LPS; Sigma) at the suboptimal concentration of 0.01 µg/ml, for a final concentration of 1 and 10 IU/ml (FSH or LH).

After a 48-h incubation at 37°C and 5% CO2, the assay plates were stored at –80°C until the ELISA was performed. For the ELISA, 200 µl of each sample (samples derived from the LPS-treated human PBMC were firstly diluted 1:20) was plated into each well of the assay plate as a singleton.

IL-2 and IL-2 receptor induction

Jurkat cells (2×10^5 cells per well) were incubated for 48 h at 37°C with or without phorbol dibutyrate (PDB; Sigma) (10 ng/ml), PHA (2 or 1 µg/ml) and the gonadotrophins at the indicated concentrations in a final volume of 200 µl per well in 96-well flat-bottomed plates. Assays were performed in duplicate; controls were performed in quadruplicate. At the end of the incubation, plates were centrifuged at 400 g for 5 min and 120 µl supernatant was collected from each well and stored at –80°C.

The cells from the culture with the highest concentration of PHA and gonadotrophins, and the relative controls, were collected from
the plate and analysed for CD25 cell surface expression (IL-2α chain receptor). The cells were labelled with 10 µl antihuman CD25 monoclonal antibody clone 2A3 [phycoerythrin (PE)-conjugate; Becton-Dickinson, Turin, Italy] or with IgG1-PE conjugate isotype control (Sigma), and incubated for 30 min in melting ice. After two washes with PBS, cells were analysed by flow cytometry (FACS Vantage, Becton-Dickinson) by gating viable cells (labelled with propidium iodide) and measuring fluorescence emission at 575 nm after excitation at 488 nm.

**IL-2 immunoassay**

For the ELISA, 100 µl culture supernatant was plated into each well of the assay plate. Before immunoassay, samples from the cultures incubated with PDB and PHA were diluted 1:4 in the same medium used for the induction (RPMI-1640 110% FBS).

Measurement of cytokine concentrations in the gonadotrophin preparations

The gonadotrophin preparations indicated in the Results section were assayed for their content of IFNγ, IL-1α, IL-1β, IL-2, IL-4, TNFα and TNFβ. The gonadotrophin vials were reconstituted with 1 ml saline and tested according to the supplier’s instructions. All preparations were tested in a single evaluation at 75 IU/ml and in duplicate at 7.5 IU/ml, except for IL-1 and IL-2 which were tested at 10 IU/ml. According to the supplier’s instructions, 200 µl such solutions was used for IL-1α, IL-1β, IL-2, IL-4, TNFα and TNFβ, while 50 µl was assayed for IFNγ.

**Statistical analysis**

Results were evaluated by analysis of variance.

**Results**

**Proliferation assays with human PBMC**

Experiments performed with PBMC from healthy subjects demonstrated various effects with the different HMG preparations. The spontaneous proliferation of PBMC was significantly increased by Humegon at the highest concentration tested ($P < 0.008$), whereas the other preparations did not modify this parameter (Figure 1). This effect with Humegon was dose-dependent ($P < 0.004$, data not shown) and not related to batch (Figure 1). In contrast, Pergonal appeared to show a batch-related effect (not significant) without dose dependency. PHA-induced proliferation was not modified by any preparation, and the responses of all of the subjects were similar to the control values (data not shown).

When PBMC were stimulated to proliferate in response to allogeneic cells in an MLR, a significantly ($P < 0.005$) increased proliferation was observed in the samples in the presence of 10 IU/ml Pergonal and Metrodin, while no consistent effects were found with the other preparations (Figure 2). The two lowest concentrations of both preparations gave results which were not significantly different from control values.

**IL-1 secretion**

The two HMG preparations tested (Pergonal and Humegon) induced the release of IL-1α and IL-1β from unstimulated PBMC from different subjects above the linear range of the immunoassay, that is $>250$ pg/ml (data not shown). This effect was not observed with Metrodin HP or with the r-hFSH product Gonal-F (below the quantitation limit of 3 pg/ml). To determine whether the presence of LH in the crude preparations was responsible for IL-1 induction, rLH was also tested and found to be ineffective. The same pattern was observed with the THP-1 human monocytic cell line, where Metrodin was also tested and found to induce IL-1β secretion ($>250$ pg/ml). Only Pergonal and Metrodin were shown to contain immunoreactive IL-1β (70 pg of IL-1β/10 IU FSH for Pergonal and 14 pg of IL-1β/10 IU FSH for Metrodin), while IL-1α was not present in any of the preparations tested.

**IL-2 secretion and IL-2 receptor expression in Jurkat cells**

Four different FSH preparations and one LH preparation were tested on Jurkat cells stimulated with PHA at two concentrations and PDB acetate. Basal production in the absence of stimuli was below the quantitation limit of the immunoassay and no gonadotrophin preparation induced measurable IL-2 production.

PDB in association with PHA stimulated the Jurkat cells to secrete IL-2, depending on the concentration of PHA. No
**Effects of urinary gonadotrophins on T cells**

Figure 2. Effect of gonadotrophin preparations (10 IU/ml) on mixed lymphocyte reaction-induced peripheral blood mononuclear cell proliferation. Stimulation index (SI) = c.p.m. sample/c.p.m. control; the means of the groups are indicated by the horizontal bars.

Table I. Interleukin-2 production (pg/ml) by Jurkat cells activated with phytohaemagglutinin (PHA) and phorbol dibutyrate (PDB)

<table>
<thead>
<tr>
<th>Gonadotrophin concentration (IU/ml)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3212</td>
<td>2262</td>
</tr>
<tr>
<td>SD</td>
<td>244</td>
<td>105</td>
</tr>
<tr>
<td>Mean</td>
<td>3627</td>
<td>2528</td>
</tr>
<tr>
<td>Ind. values</td>
<td>3599, 3565</td>
<td>2532, 2523</td>
</tr>
<tr>
<td>Mean</td>
<td>3049</td>
<td>2427</td>
</tr>
<tr>
<td>Ind. values</td>
<td>2456, 3643</td>
<td>2239, 2515</td>
</tr>
</tbody>
</table>

IL-2 secretion was induced with PHA 2 µg/ml + PDB 10 ng/ml (A) or with PHA 1 µg/ml + PDB 10 ng/ml (B). Controls are shown as mean ± SD of four determinations; the mean of two determinations and individual values are shown for gonadotrophin preparations. Ind. = individual.

major modification in IL-2 secretion, except a decreasing trend for Humegon, was observed (Table I).

As regards CD25 (IL-2 receptor α chain) expression, no major modifications were induced by any of the gonadotrophins, either alone or in association with PDB and PHA (data not shown).

**Evaluation of cytokine content in different HMG preparations**

Different batches of 11 commercially available HMG preparations were measured by immunoassay for the presence of IFNγ, IL-4, TNFα and TNFβ. None of these cytokines was found in any of the products (data not shown).

**Discussion**

Local reactions to gonadotrophin administration have been reported (Li and Hindle, 1993; Harika et al., 1994; Odink et al., 1995; Redfearn et al., 1995; Albano et al., 1996). These adverse reactions are probably mediated immunologically, with some representing classic IgE-mediated reactions, such as urticaria and angio-oedema, which are usually the result of protein antigens (Stites et al., 1987). The proteins co-administered as impurities with conventional gonadotrophin preparations might increase the rate of allergic reactions in the recipients, even if all of the proteins were of human origin. It is documented that exogenously administered human proteins can induce immune reactions in patients, as described for example with IFN preparations (see Antonelli, 1994 for references), insulin (Fineberg et al., 1983), human growth hormone (Zeisel et al., 1992), HCG (Braunstein et al., 1983) and LH (Healy et al., 1978). Moreover, Harika and colleagues (1994) have reported anaphylactic shock in a woman undergoing stimulation for IVF with urinary HMG. In this woman, a skin prick test showed a reaction only with the same preparation used 2 years earlier for a previous IVF cycle, while a product from a different manufacturer did not induce a skin reaction. As the active ingredient was the same in the two HMG preparations, this case report indirectly confirms the relevance of extraneous non-gonadotrophin proteins to the risk of allergic reactions. Similarly, a patient who had shown an immediate-
type reaction using HMG was treated without allergic symptoms with rFSH (Albano et al., 1996). The same conclusion can be drawn from recent case reports showing that delayed-type hypersensitivity reactions to HMG were avoided by using highly purified hFSH in women with allergic reactions to ‘crude’ preparations (Li and Hindle, 1993; Redfearn et al., 1995).

Recently, a highly purified human urinary FSH preparation (Metrodin HP) became available in which HFSPHSH represents >95% of the total protein content (Le Cotonnec et al., 1993) (approximately 10 000 IU FSH/mg protein). The use of Metrodin HP is expected to decrease the risk of allergic reactions because of the negligible content of contaminating proteins compared with other urinary gonadotrophin preparations.

Several human urinary proteins have been identified by Giudice et al. (1994) using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting with urinary HMG/FSH preparations from different producers. All ‘crude’ urinary products tested, including those employed in the present study, showed protein bands in addition to that of FSH. There was also a certain degree of variability between batches of the same product. Only Metrodin HP showed a single band recognized as FSH by Western blotting.

Among the contaminating proteins found in the crude preparations, some reacted with specific antisera used in Western blotting. They were present either in all of the products (p55 TNF receptor, transferrin, IgM, Ig-light chains) or in some of them (Ig fragments, Tamm-Horsfall glycoprotein, urokinase). Many of these reaction products were found as bands with a different molecular mass to that of the intact proteins, suggesting that they were degraded (Giudice et al. 1994). The same authors showed, by ELISA, that Humegon contained measurable amounts of epithelial growth factor, while no IL-6 was seen in any of the products.

The present study demonstrates that some of the gonadotrophin preparations modulate parameters of immune function in vitro. However, different products gave a substantially different pattern of activity. Gonal-F and Metrodin HP, which are pure hFSH preparations, did not show any effect in any of the tests performed, thus ruling out the possibility that the effects observed with the other preparations were caused by hFSH. Metrodin and Pergonal increased lymphoproliferation in MLR. As IL-1 is known to enhance the antigen-presenting function, this effect might be explained by the presence of IL-1β as a contaminant in these preparations or by the induction of both IL-1α and β secretion from PBMC. Humegon induced a proliferative response of PBMC in the absence of other stimuli, but decreased PHA-induced IL-2 production. Other stimulatory mediators could be present, but immunossays excluded the presence of IL-2, IL-4 or IFNγ. It can be postulated that, consistent with SDS–PAGE and immunoblotting (Giudice et al., 1994), some degradation of the contaminating proteins had occurred during purification procedures, and that some antigenic determinants may have been produced that can trigger PBMC proliferation.

None of the cytokines that can induce allergic-like reactions per se, and for which an immunoassay was performed (IL-4, TNFα and β, IFNγ), were found in the crude HMG preparations with the exception of IL-1 (see above). It is therefore likely that at least some of the reactions observed in patients are caused by protein contaminants recognized as allergens. In addition, allergic-like reactions could be caused by the presence of unidentified substances that directly provoke histaminergic-like reactions in susceptible patients. Furthermore, this study demonstrates that crude urinary HMG/FSH preparations from different manufacturers have different in-vitro immunological effects, reflecting a different profile of contaminants.

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References

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