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## Effect of Imiquimod on Cytokine Induction in First Trimester Trophoblasts

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### ABSTRACT

**Objectives:** Imiquimod (IQ) is used clinically for the topical treatment of external genital warts. IQ is an immune response modifier and induces the expression of interferon- $\alpha$  and other cytokines in human Peripheral Blood Monocytes (PBMC). Trophoblasts have been previously shown to express inflammatory cytokines upon lipopolysaccharide (LPS) stimulation. The objective of this study was to evaluate the ability of IQ to induce transcription of cytokines in trophoblasts.

**Methods:** A transformed human first trimester trophoblast cell line, HTR-8/SVneo, was cultured in DMEM containing IQ at concentrations of 0 to 5.0  $\mu\text{g/ml}$ . 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assays were conducted to control for any drug-induced cell death. Total RNA was isolated from trophoblasts at 0, 8 and 24 hours of culture and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was conducted using specific amplimers for the inflammatory cytokines interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8. RT-PCR of  $\beta$ -actin was performed to control for equal RNA loading.

**Results:** RT-PCR was unable to detect an increase in either IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 or IL-8 mRNA in first trimester trophoblasts cultured in the presence of 0 to 5.0  $\mu\text{g/ml}$  of IQ for up to 24 hours. RT-PCR confirmed equal RNA loading and MTT viability assays did not show loss of cell viability at concentrations of IQ up to 5.0  $\mu\text{g/ml}$ .

**Conclusions:** IQ, at the concentrations tested, did not induce the transcriptional expression of inflammatory cytokines in human first trimester trophoblasts. These data suggest that IQ would not induce the expression of inflammatory cytokines in placental trophoblasts. *Infect. Dis. Obstet. Gynecol.* 8:105–111, 2000. © 2000 Wiley-Liss, Inc.

### KEY WORDS

imiquimod; trophoblast; cytokines; herpes; immunomodulation

Imiquimod (IQ), [1(2-methylpropyl)-1h-imidazo[4,5-c]quinolin-4-amine], belongs to a class of imidazoquinolines with immunomodulatory characteristics. IQ is commonly used in the treatment of genital warts. This heterocyclic amine, which has potent antiviral and antitumor activity, stimulates the *in vitro* production of interferon- $\alpha$  (IFN- $\alpha$ ), interleukin-1 $\alpha$  (IL-1 $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), in-

terleukin-6 (IL-6) and interleukin-8 (IL-8).<sup>1–3</sup> Mononuclear phagocytes have been shown to be the cell type that is largely responsible for the expression of these cytokines following treatment with IQ.<sup>2–3</sup> The induction of these cytokines is through transcriptional activation of cytokine-specific mRNA's.<sup>1</sup> Placental trophoblasts share many phenotypic similarities with monocytes and

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Clinical Study

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may therefore be affected by the immunomodulatory characteristics of IQ.

The human placenta is a multifunctional endocrine organ that serves as both a mechanical and immunological barrier against the vertical transmission of infectious agents. Placental trophoblasts are critical components of this barrier and have been shown to express a variety of both proinflammatory and immunoregulatory cytokines including IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, when stimulated.<sup>4</sup> These cytokines presumably function to elicit and modulate inflammatory immune responses, promote trophoblast differentiation and aid in maintaining normal placental function.<sup>5</sup> Use of this drug during pregnancy could potentially alter the normal cytokine milieu at the maternal-fetal interface and therefore adversely affect trophoblast and hence placental function. This study was conducted to determine if IQ is capable of altering the transcriptional expression of immunomodulatory cytokines in human first trimester trophoblasts *in vitro*.

## SUBJECTS AND METHODS

### Cell Culture

HTR-8/SVneo cells,<sup>6</sup> derived from human first trimester extravillous trophoblasts, possess a normal trophoblast morphology and express cytokeratin, human chorionic gonadotrophin and type IV collagenase. Cells were grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM) (SIGMA, St. Louis, MO), supplemented with 10% non-heat inactivated fetal bovine serum, 1% L-glutamine, 50 U/mL penicillin and 5  $\mu$ g/mL streptomycin (GIBCO BRL, Gaithersburg, MD). Cells were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> in 75 cm<sup>2</sup> flasks (VWR Scientific Products, Chicago, IL).

### Experimental Culture Conditions and RNA Isolation

Confluent HTR-8/SVneo monolayers were washed three times in 4 mL of DMEM prior to culture under experimental conditions. Cells were subsequently cultured in media alone or in the presence of various concentrations (0 to 5.0  $\mu$ g/mL) of IQ for 0, 8 or 24 hours. At each time point, total RNA was isolated from cells using the TRIzol<sup>®</sup> Reagent (GIBCO BRL, Gaithersburg, MD), in accordance with the manufacturers' instructions. RNA integrity was assessed by agarose gel electrophoresis and quantitated spectrophotometrically at 260/280 nm.

Contaminating genomic DNA was digested with 4 U/sample of RQ1 DNase in 1 $\times$  DNase buffer (Promega, Madison, WI), for 1.5 hr at room temperature. This trophoblast cell line has been shown previously to express various inflammatory cytokines following lipopolysaccharide induction.<sup>4</sup>

### Cell Viability Assay

Cell viability in media alone or in the presence of IQ (0 to 5.0  $\mu$ g/mL), was examined following 0, 8 and 24 hours of culture. A rapid colorimetric assay utilizing the tetrazolium salt of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (SIGMA), was conducted as described previously.<sup>7</sup> Assays were performed in triplicate using a 96-well microtitre plate format and absorbances were determined using a plate-reading spectrophotometer (Ceres UV900HDI, Bio-TEK Instruments Inc., Winooski, VT) at a reference wavelength of 630 nm and a test wavelength of 570 nm.

### Reverse Transcription-Polymerase Chain Reaction

RT-PCR amplification was conducted using amplimers to IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and  $\beta$ -actin (IDT Technologies, Coralville, IA). Sequence data for the construction of amplimers was obtained from the public domain. Total RNA (1  $\mu$ g) was reverse transcribed and the cDNA amplified in a 50  $\mu$ L reaction volume containing 1 $\times$  buffer (Perkin Elmer Applied Biosystems, Norfolk, CT), 0.5 mM sense and anti-sense primers, 2.0 mM Mn(OAc)<sub>2</sub>, 0.15 mM dNTPs, and 5 U/reaction of rTth enzyme (Perkin Elmer Applied Biosystems, Norfolk, CT). The PCR reaction was overlaid with 50  $\mu$ L of sterile mineral oil and reverse transcription occurred for 60 min at 60°C followed by a denaturation step at 95°C for 2 min. PCR amplification was performed at a denaturation temperature of 95°C for 45 sec and an annealing temperature of either 52°C (IL-1 $\beta$ ) or 55°C (IL-1 $\alpha$ , IL-6, IL-8 and  $\beta$ -actin), for 45 sec and 35 cycles. This was followed by a terminal extension cycle at 72°C for 15 min. Reverse transcription and PCR amplification were conducted in a Perkin Elmer 480 DNA thermocycler (Perkin Elmer Applied Biosystems). PCR products were resolved by agarose gel electrophoresis and product sizes were estimated by comparison with a molecular size standard (Pvu II digest of  $\lambda$  bacte-

riophage DNA).  $\beta$ -actin amplimers and where possible, cytokine amplimers, were designed to span intronic regions in order to control for the presence of contaminating genomic DNA. Levels of cytokine and  $\beta$ -actin mRNA were titrated by serial dilution prior to reverse transcription, in order to remain within the linear range of the PCR amplifications (data not shown). All reaction sets included negative PCR and negative RNA controls.

## RESULTS

HTR-8SVneo cell viability was examined following 0, 8 and 24h of culture in the presence of IQ. No concentration-dependent or time-dependent change in cell viability was detected during the course of this study (Fig. 1). Total RNA was isolated from HTR-8SVneo cells grown under experimental and control conditions for RT-PCR. RNA integrity was confirmed by agarose gel electrophoresis (Fig. 2). RT-PCR amplification of  $\beta$ -actin was conducted to ensure equal RNA loading throughout the experiment. RT-PCR analysis demonstrated the appearance of a single 309bp  $\beta$ -actin product of essentially equal intensity among all experimental and control sets (Fig. 3). RT-PCR analysis was subsequently conducted to determine the relative mRNA levels of specific immunomodulatory and proinflammatory cytokines. RT-PCR amplification using amplimers to IL-1 $\alpha$  (Fig. 4), IL-1 $\beta$  (Fig. 5), IL-6 (Fig. 6) and IL-8 (Fig. 7), yielded discrete products of 500bp, 398bp, 494bp and 217bp, respectively. No concentration-dependent or time-dependent effect of IQ on cytokine mRNA levels in human trophoblasts was noted.

## DISCUSSION

IQ is safe and effective for the topical treatment of genital warts.<sup>8-9</sup> The anti-viral nature of this drug is attributed largely to the induced expression of IFN- $\alpha$  and other immunomodulatory cytokines by monocytes.<sup>1</sup> Trophoblasts, which are phenotypically similar to monocytes, are known to express immunomodulatory and proinflammatory cytokines following induction by infection stimuli.<sup>4</sup> This study examined the ability of IQ to induce the *in vitro* expression of similar cytokines in first trimester trophoblasts. Such induction may be contraindicated during pregnancy because exposure to an altered cytokine milieu could adversely affect

## Cell Viability Assay

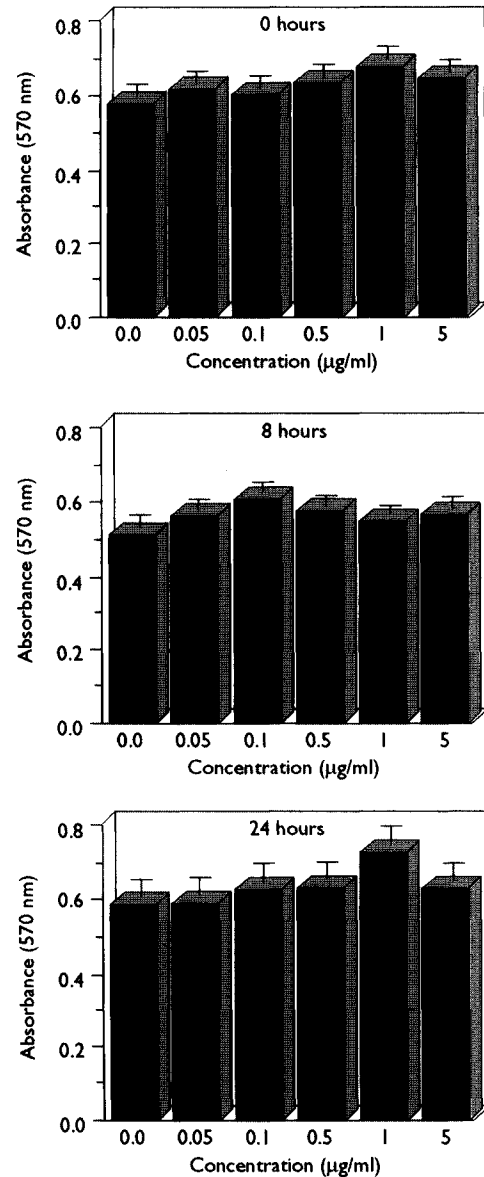


Fig. 1. HTR-8SVneo cell viability assay. Viability of first trimester trophoblasts was evaluated following culture in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 or 5.0  $\mu$ g/mL) for 0h (top), 8h (middle) and 24h (bottom).

trophoblast function or fetal immune development.<sup>10-11</sup>

RT-PCR demonstrated neither a concentration-dependent nor time-dependent induction of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 or IL-8 in first trimester trophoblasts by IQ. The presence of RNA transcripts to these cytokines in trophoblasts cultured under control conditions is consistent with previous observations suggesting that these cytokines may be ex-

## RNA- Integrity

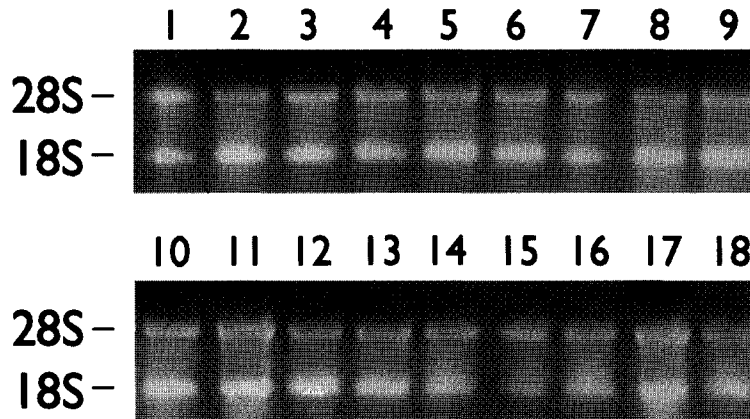


Fig. 2. Determination of RNA integrity. Total RNA was isolated from first trimester trophoblasts cultured in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 and 5.0 µg/mL) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

## β-Actin

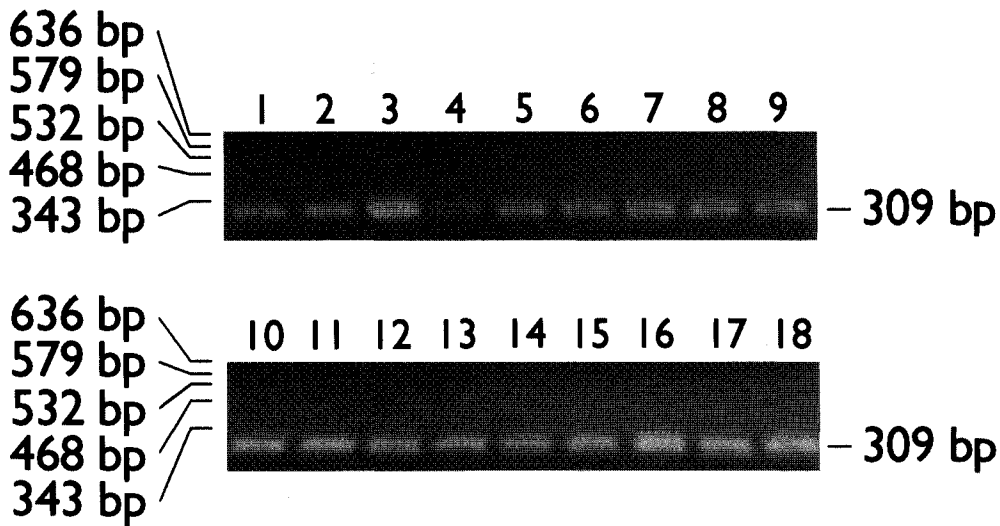


Fig. 3. Transcriptional expression of β-actin in trophoblasts. Human β-actin was amplified from trophoblasts to control for equal loading of RNA. RT-PCR amplification yielded a single 309bp product of nearly equal intensity from trophoblasts cultured in the presence of IQ (0, 0.1, 0.5, 1.0 and 5.0 µg/mL) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

pressed at a low constitutive level.<sup>4</sup> These results are also consistent with previous observations suggesting that IQ is largely immunomodulatory for antigen presenting cells such as monocytes and

macrophages. Resident macrophages and monocytes present within the placenta may therefore respond to IQ treatment by expression of cytokines. These data support the hypothesis that IQ

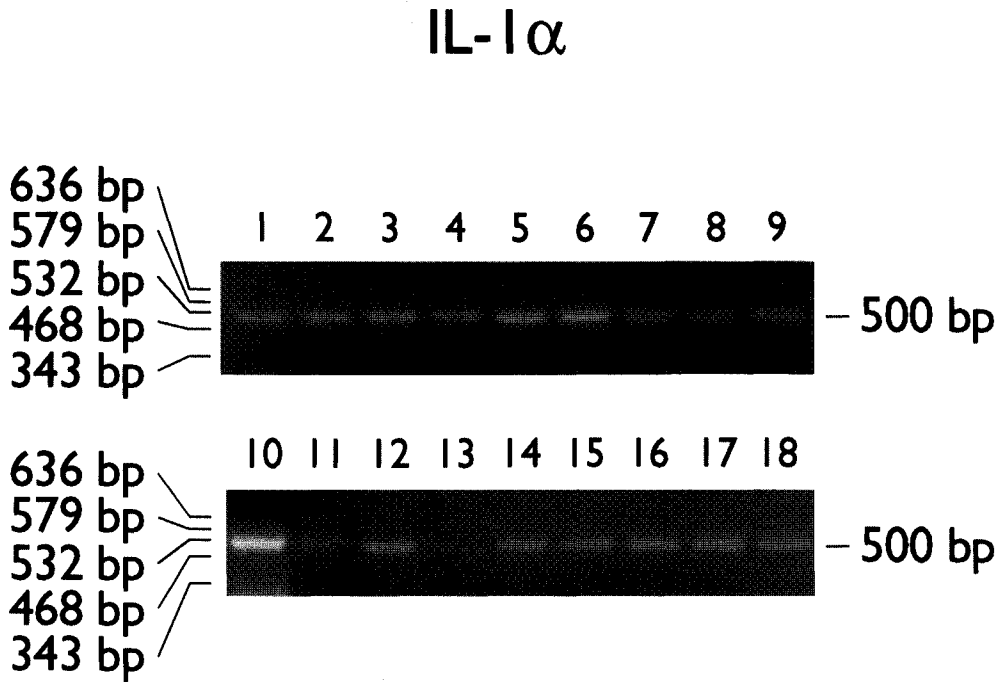


Fig. 4. Transcriptional expression of IL-1 $\alpha$  in trophoblasts. RT-PCR yielded a single 500bp product of nearly equal intensity from trophoblasts cultured in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 and 5.0  $\mu$ g/mL) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

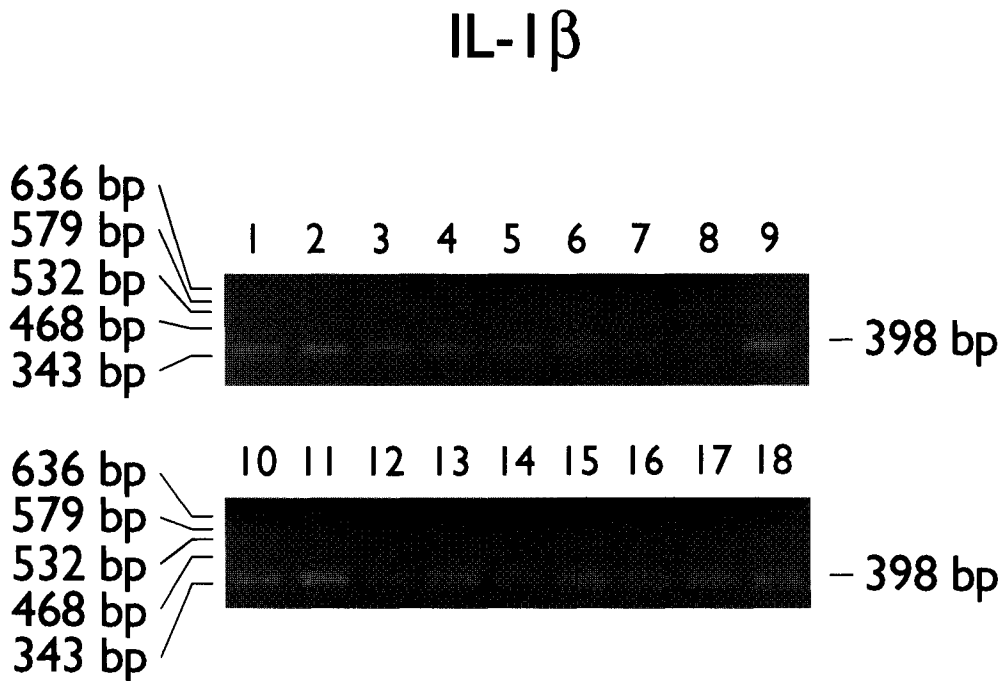


Fig. 5. Transcriptional expression of IL-1 $\beta$  in trophoblasts. RT-PCR yielded a single 398bp product of nearly equal intensity from trophoblasts cultured in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 and 5.0  $\mu$ g/mL) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

## IL-6

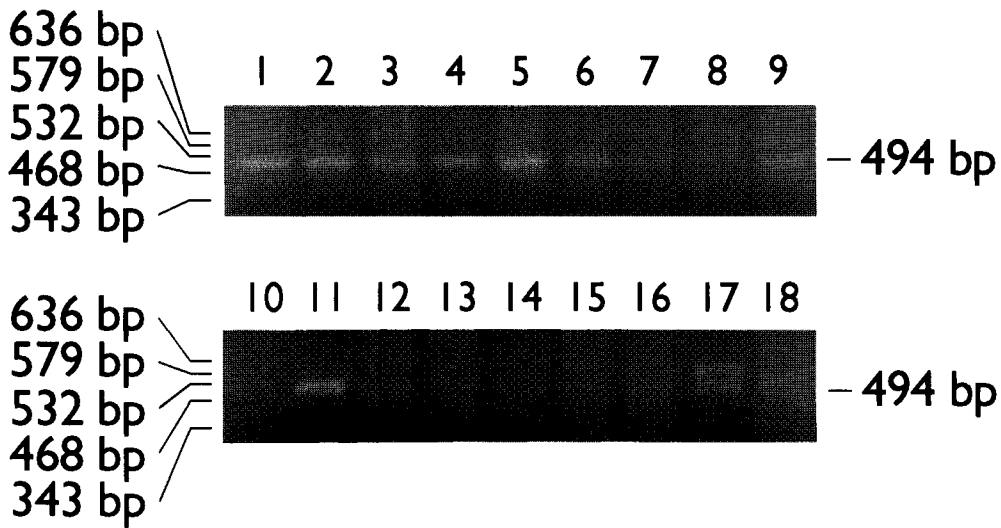


Fig. 6. Transcriptional expression of IL-6 in trophoblasts. RT-PCR yielded a major 494bp product from trophoblasts cultured in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 and 5.0  $\mu\text{g}/\text{mL}$ ) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

## IL-8

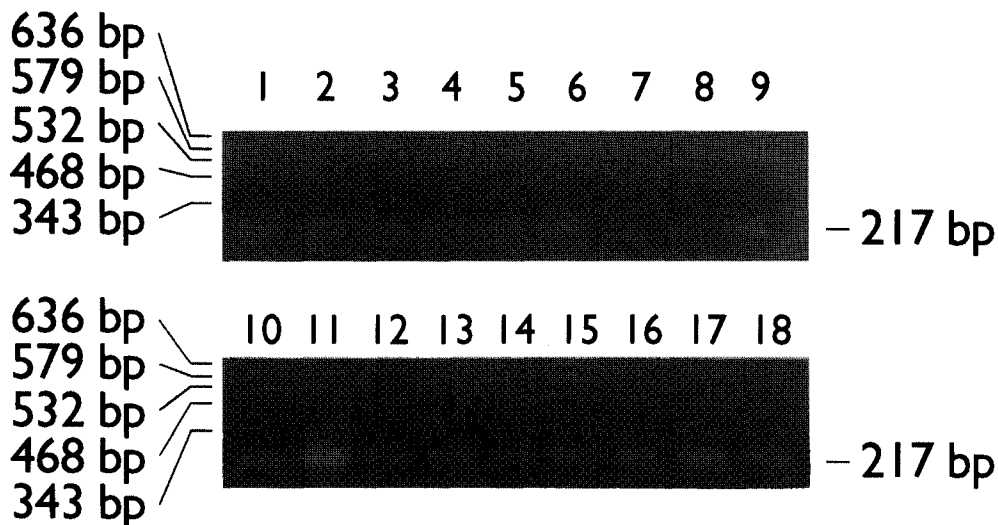


Fig. 7. Transcriptional expression of IL-8 in trophoblasts. RT-PCR yielded a single 217bp product of nearly equal intensity from trophoblasts cultured in the presence of IQ (0, 0.05, 0.1, 0.5, 1.0 and 5.0  $\mu\text{g}/\text{mL}$ ) for 0h (lanes 1–6), 8h (lanes 7–12) and 24h (lanes 13–18).

does not transcriptionally induce specific proinflammatory and immunomodulatory cytokines in placental trophoblasts.

### CONCLUSIONS

Imiquimod does not induce the *in vitro* transcription of the immunomodulatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 or IL-8 in human first trimester trophoblasts. Imiquimod is therefore unlikely to alter expression of these cytokines in the placenta. The inability of imiquimod to transcriptionally induce cytokines in trophoblasts at the concentrations tested resolves some of the concerns associated with administering imiquimod during pregnancy. Imiquimod, at the levels tested, does not contraindicate use during pregnancy in humans. Further evaluation *in vivo* will be necessary to confirm the safety and efficacy of imiquimod during pregnancy.

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