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Enhancement of the innate and cellular immune response in patients with genital warts treated with topical imiquimod cream 5%

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Abstract

The mechanism of action of imiquimod 5% cream applied topically to patients with genital warts was evaluated in a double-blind, placebo-controlled study. Imiquimod (16 patients) or placebo (three patients) was applied three times per week for up to 16 weeks. All imiquimod-treated patients had a $\geq 75\%$ reduction in total wart area while only one of three placebo-treated patients had a similar reduction. Wart biopsies were taken at prestudy, week 6, and end of treatment. Polymerase chain reaction (PCR) for human papillomavirus (HPV) DNA and reverse transcriptase (RT)-PCR for messenger (m)RNAs were used to identify cytokines, cellular markers, viral gene products, and cell cycle markers in these biopsies. Treatment with imiquimod, an immune response modifier, stimulated significant increases in mRNA for interferon (IFN)-α, IFN-γ and 2′,5′ oligoadenylate synthetase (2′,5′-AS) as well as a tendency towards increases in tumor necrosis factor (TNF)-α and interleukin-12 p40. Significant increases in mRNA for CD4 and a trend toward increases in CD8 were also observed in imiquimod-treated patients, suggesting activation of a cell mediated immune response. Imiquimod administration was also associated with a significant decrease in viral load as measured by HPV DNA and L1 mRNA. The effects on HPV markers were accompanied by an apparent decrease in mRNA expression for markers of cell proliferation and an increase in mRNA for markers of keratinocyte differentiation and tumor suppressors.

Keywords: Aldara™; Cytokine; Condylomata acuminata; Human papilloma virus; Imiquimod; Interferon

Abbreviations: 2′,5′-AS, 2′,5′ oligoadenylate synthetase; CMI, cell mediated immunity; CTL, cytotoxic T-cells; EOT, end of treatment; HPV, human papillomavirus; IFN, interferon; IL, interleukin; LC, Langerhans cell; mRNA, messenger RNA; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction; Th, T helper; TNF-α, tumor necrosis factor-α.

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1. Introduction

Human papillomavirus (HPV) infection is the most common sexually transmitted viral disease (Becker et al., 1987). Patients infected with HPV (primarily types 6 and 11) can develop anogenital warts (condylomata acuminata), and it is estimated that 1–2% of the sexually active population between the ages of 15 and 49 is afflicted with these HPV-associated verrucae (Koutsky et al., 1988). A variety of methods is currently used for the treatment of anogenital warts; however, the clearance results and recurrence rates are less than satisfactory (Kraus and Stone, 1990; Stone, 1995).

Recently, Aldara™ (imiquimod, R-837, or S-26308) cream 5% became available for the treatment of patients with external genital and perianal warts. Phase III clinical studies have demonstrated that this therapy is significantly more effective than vehicle (placebo) in clearing genital/perianal warts (Beutner et al., 1998; Edwards et al., 1998). Of the patients who cleared their baseline warts, only 13% of the patients had a recurrence of their warts at 12 weeks of follow-up, suggesting either eradication of the virus or immunologic control of the infection. The second hypothesis seems more likely since in a previous analysis of imiquimod-treated condyloma, reduction but not elimination of HPV DNA was observed (Tyring et al., 1998).

The effectiveness of imiquimod in treating this disease is thought to be mediated by activation of components of the innate and cell mediated arm of the acquired immune response, because the drug has no direct antiviral, antiproliferative, or cytodestructive properties (Harrison et al., 1988; Kono et al., 1994). Imiquimod's acute antiviral and antitumor activity in animals is due to its activation of the innate immune response through the production of cytokines, including interferon (IFN)-α (Harrison et al., 1988, 1994; Sidky et al., 1992; Reiter et al., 1994; Megyeri et al., 1995; Testerman et al., 1995; Tomai et al., 1997). Preclinical studies have also shown cytokine induction after systemic drug administration, as well as local induction of IFN-α and tumor necrosis factor (TNF)-α in the skin of mice and rats following topical application (Harrison et al., 1994; Reiter et al., 1994; Tomai et al., 1997; Imbertson et al., 1998). Biopsies taken from patients whose genital warts were regressing also had significantly higher levels of messenger ribonucleic acid (mRNA) for both IFN-α and TNF-α than those patients showing little or no regression (Tyring et al., 1998). These cytokine results argue strongly for a role of enhancement of the innate immune response in mediating imiquimod's antiviral and antiproliferative activities.

A number of the cytokines known to be induced by imiquimod can also affect acquired immune responses. Interferon-α is capable of potentiating the cellular arm of the immune response, while inhibiting the humoral arm (Parronchi et al., 1992; Schandene et al., 1996). In a number of animal models, imiquimod potentiates acquired immunity, specifically the cellular arm of the response (Bernstein and Harrison, 1989; Bernstein et al., 1993; Harrison et al., 1994). Most recently, studies with imiquimod have demonstrated direct induction of interleukin (IL)-12 and subsequent indirect induction of IFN-γ (Wagner et al., 1999), two additional cytokines known to be important in generating and maintaining a cell mediated immune (CMI) response. A durable effect on HPV-specific CMI by imiquimod may in part explain the low recurrence rates reported in clinical trials (Beutner et al., 1998; Edwards et al., 1998).

In a previous report on the mechanism of action of imiquimod in patients with genital warts, clearance of warts was associated with significant increases in mRNA expression for IFN-α, IFN-β, IFN-γ, and TNF-α (Tyring et al., 1998). Wart reduction was also associated with a significant decrease in HPV DNA copy number/cell as well as mRNA expression for HPV early (E7) and late (L1) proteins. In that report, patients were grouped into those that showed a greater than 75% reduction in wart area and those that did not. In the analysis reported herein, we further examine the effects of topically-applied imiquimod or vehicle on mRNA expression for cytokines, various cell surface markers, HPV markers and markers of proliferation and differentiation. Semiquantitative PCR was used to determine relative mRNA expression for the various

markers in biopsies obtained before, during, and after treatment, and PCR was used to determine HPV DNA copy number/cell. In this report, patients were grouped into those treated with imiquimod or those treated with placebo regardless of the clinical outcome.

2. Materials and methods

2.1. Patient selection

Patients were 18 years of age or older with a histologically confirmed diagnosis of condylomata acuminata. Patients were excluded if they were known to be positive for human immunodeficiency virus, or had high-grade cervical intraepithelial lesions. In addition, those patients who had previously been treated with imiquimod or who, within 4 weeks of the study, had received IFN, an IFN-inducer, an immunomodulator, oral or topical antiviral drugs, cytotoxic or investigational drugs, or chemical and/or surgical wart therapy were excluded. Patients who received any topical non-wart therapy to the wart site, or oral or inhaled corticosteroids (>1000 μg/day) within 2 weeks of the study were also ineligible. Patients had to have at least ten but no more than 50 warts prior to prestudy biopsy. Some components of this study have been previously published (Tyring et al., 1998).

2.2. Study design

This was a randomized, double-blind, vehicle-controlled study conducted in 22 patients (12 males and ten females). Patients were enrolled after study procedures were explained, and they signed written informed consent. Patients self-applied either imiquimod 5% (Aldara™) or vehicle (placebo) cream to their warts overnight (8 ± 2 h) three times per week (non-consecutive days) for a maximum of 16 weeks. Study drug was randomized at a ratio of 4:1 for each gender, so that for every four patients who received imiquimod cream, one received vehicle. The patients returned to the clinic every 2 weeks until their target genital/perianal warts cleared or through 16 weeks of treatment, whichever occurred first.

A biopsy of the target wart area was obtained by scissor or scalpel excision prior to treatment to confirm condylomata acuminata and to establish a baseline for the biologic markers. Biopsies were taken again at treatment week 6 and at end of treatment (EOT). In patients whose baseline warts totally cleared, the end of treatment biopsy was taken from the same anatomic site as the prestudy and treatment week 6 biopsies. If $>40\%$ clearance of wart tissue was observed earlier than treatment week 6, a biopsy was taken at that time rather than at 6 weeks and was designated as the week 6 biopsy. All biopsies were flash frozen in liquid nitrogen and stored at -70°C until analyses were performed.

2.3. Detection and typing of HPV

Genomic DNA was isolated from biopsies by Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's specifications. DNA (500 ng) was amplified by PCR, using consensus HPV L1 primers simultaneously with β-globin primers as described earlier (Arany et al., 1995c). HPV PCR products were either hybridized with HPV 6 or 11 type-specific probe or sequenced for type identification (Rady et al., 1993).

2.4. Estimation of HPV DNA copy numbers

HPV DNA copy number/cell was estimated by comparing the intensity of the amplification product of the HPV gene with the β-globin gene. The normalized intensity was then compared to amplification products obtained from DNA of HPV-positive cell lines or cloned HPV DNA to obtain the estimate of the number of HPV copies/cell (Arany et al., 1995b).

2.5. RT-PCR for specific mRNAs

Total RNA was isolated from the wart tissue using Tri-Reagent simultaneously with genomic DNA as described above. Semiquantitative reverse transcriptase (RT)-PCR was used to determine relative mRNA levels (Tyring et al., 1998). Target mRNA levels were normalized to the levels

of glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) for statistical analysis and treatment comparisons. The primer pairs for G3PDH, TNF-α, IFN-α, IFN-β, IFN-γ, IL-2, IL-4, IL-5, CD4, CD8 and c-myc were purchased from Clontech (Palo Alto, CA), while those for HPV type 6 and 11-specific E7 and L1, 2′,5′-AS, IL-12 p40, CD1a, CD29, CD45Ro, CD14, CD16, 2′,5′-AS, p53, proliferating cell nuclear antigen (PCNA), the retinoblastoma gene product (pRb), filaggrin, and the keratinocyte protein (K10) were designed and synthesized by BioSynthesis, Denton, TX (Arany et al., 1995a,c; Arany and Tyring, 1996a; Tyring et al., 1998).

2.6. Statistical methods

For each marker, the ratio of the marker to G3PDH was computed at prestudy (baseline), week 6, and end of treatment. The ratio change from baseline was computed at week 6 and end of treatment for each patient by taking the ratio of marker/G3PDH at week 6 and end of treatment and dividing by the baseline value. A Wilcoxon rank sum test compared this ratio change between placebo- and drug-treated patients at each time-point. A Wilcoxon rank sum test also compared the baseline ratios of each marker to G3PDH between treatment groups in order to test baseline equality. Spearman rank coefficients of correlation were also used to measure the association between predefined markers that were expected to change in tandem.

Since this study was designed to explore which of many markers are associated with imiquimod treatment and wart reduction, no adjustment to the type I error rate was done. Any P-value less than 0.05 was considered significant.

3. Results

3.1. Effects on cytokine mRNA expression

A total of 22 patients was randomized into the study and three were lost to follow-up prior to obtaining the week 6 biopsy; therefore this report is limited to 19 patients, 16 receiving imiquimod and three receiving placebo. All imiquimod-treated patients and one of three placebo-treated patients had $\geq 75\%$ clearance of their baseline warts. Biopsy analysis included all 19 patients at week 6 and 17 patients at the end of treatment (14 receiving drug and three receiving placebo). As previously reported, wart regression was associated with a significant increase in cytokines (IFN-α, IFN-β, IFN-γ and TNF-α) known to be activated by imiquimod and a significant reduction in markers for viral infection (HPV DNA, L1 mRNA and E7 mRNA) (Tyring et al., 1998). In this previous report, analyses focused on markers that were associated with wart regression regardless of treatment and for analyses purposes the patient who cleared their baseline warts while receiving placebo was grouped with imiquimod treated patients. The analysis described herein compares the effects observed during and following imiquimod and placebo treatment regardless of treatment outcome. The levels of specific markers for the patient who cleared while receiving placebo are also included.

The normalized baseline values for each marker were compared between vehicle and imiquimod-treated patients. None of the markers for the two groups were significantly different at baseline.

Changes in mRNA for a number of cytokines, including IFN-α, IFN-β, TNF-α, IFN-γ and IL-12 p40, and the IFN-inducible gene product, 2′,5′-AS, are summarized for both vehicle- and imiquimod-treated patients (Fig. 1). Levels of mRNA for IFN-α ($P < 0.02$), IFN-γ ($P < 0.048$) and 2′,5′-AS ($P < 0.0086$) were significantly elevated in week 6 biopsies from imiquimod treated patients compared to vehicle patients. Significant increases in mRNA for IFN-γ ($P < 0.027$) were also seen at the end of treatment. IFN-β, TNF-α, and IL-12 p40 mRNA levels tended to increase in imiquimod-treated patients at both 6 weeks and end of treatment, but these increases were not statistically significant when compared to vehicle-treated patients. Low levels of mRNA for IL-4 or IL-5 were detected in these biopsy specimens; however, no elevations over baseline were detected in either group (data not shown). Interestingly, the placebo patient who showed regression of their warts had high levels of IFN-β mRNA

expression at week 6 and end of treatment when compared to baseline.

3.2. Effects on mRNA for cell surface markers

The elevations in cytokines induced by imiquimod have the potential to effect immune cell infiltration and activation at the biopsy site. Therefore, mRNA levels for Th cells expressing CD4, cytotoxic T-cells (CTL) expressing CD8, resident Langerhans cells (LC) expressing CD1a, monocytes expressing CD14, natural killer cells expressing CD16, and cells expressing the immune activation markers CD29 and CD45Ro were evaluated. Results in Fig. 2 show a trend towards increases in CD4 mRNA levels at week 6 in imiquimod-treated patients that was statistically significant at end of treatment ($P < 0.02$). There was also a trend towards increased CD8 mRNA, CD29 mRNA and CD45Ro mRNA and decreased CD1a mRNA in the imiquimod-treated group but these changes were not significant. There were no increases in mRNA levels for CD14 and CD16 at either timepoint (data not shown). The patient who cleared their warts while receiving the placebo showed elevations in CD8 mRNA and decreases in CD1a mRNA expression at both week 6 and end of treatment.

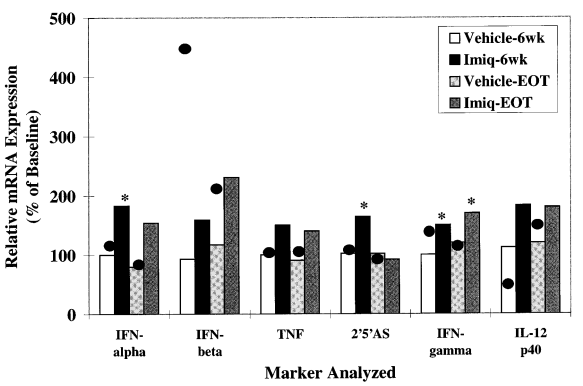

Fig. 1. Wilcoxon rank sum test comparing the ratio changes from baseline for each cytokine marker between imiquimod (imiq)-treated patients and vehicle-treated patients at both week 6 and end of treatment (EOT). $^*P \leq 0.05$. The P-values for IFN-α at week 6, for 2′,5′-AS at week 6, for IFN-γ at week 6, and IFN-γ at end of treatment were 0.02, 0.0086, 0.048 and 0.027, respectively. ● indicates results for the placebo-treated patient who cleared their warts.

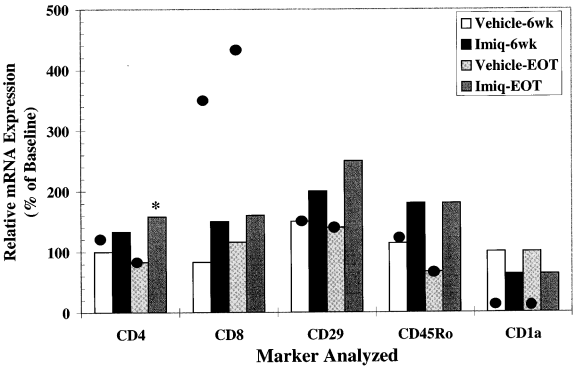

Fig. 2. Wilcoxon rank sum test comparing the ratio changes from baseline for cell surface markers between imiquimod (imiq)-treated patients and vehicle-treated patients at both week 6 and end of treatment (EOT). $^*P \leq 0.05$. The P-value for CD4 at week 6 was 0.02. ● indicates results for the placebo-treated patient who cleared their warts.

3.3. Effects on markers of viral infection

In a previous analysis of the data, wart regression was found to be associated with significant reductions in HPV DNA, L1 mRNA and E7 mRNA expression (Tyring et al., 1998). Results comparing imiquimod versus vehicle treated patients presented in Fig. 3 show a decline of HPV DNA at week 6 when compared to vehicle-treated patients. The imiquimod-treated patients also had

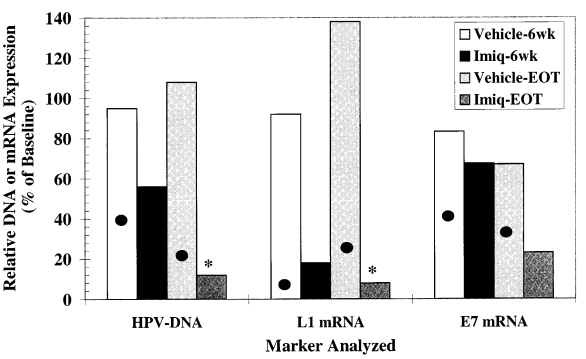

Fig. 3. Wilcoxon rank sum test comparing the ratio changes from baseline for HPV markers between imiquimod (imiq)-treated patients and vehicle-treated patients at both week 6 and end of treatment (EOT). $^*P \leq 0.05$. The P-values for HPV DNA at week 6 and for L1 mRNA at the end of treatment were 0.0376 and 0.0375, respectively. ● indicates results for the placebo-treated patient who cleared their warts.

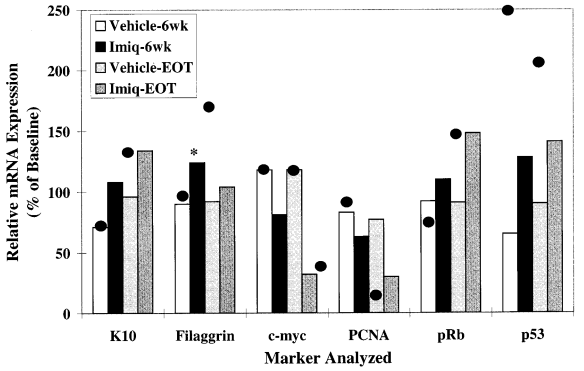

Fig. 4. Wilcoxon rank sum test comparing the ratio changes from baseline for keratinocyte and cell cycle markers between imiquimod (imiq)-treated patients and vehicle-treated patients at both week 6 and end of treatment (EOT). $*P \leq 0.05$. The P-value for filaggrin at week 6 was 0.029. ● indicates results for the placebo-treated patient who cleared their warts.

a greater than 90% median reduction in HPV DNA from baseline at the end of treatment, which was significantly reduced when compared to vehicle-treated patients ($P < 0.038$). In addition, a decline in L1 was seen at week 6 and was significantly decreased at end of treatment ($P < 0.038$). Following imiquimod treatment, there was no apparent change in E7 mRNA expression at week 6; however, E7 was reduced at end of treatment, but the reduction was not significant when compared to vehicle-treated patients. The vehicle-treated patient who cleared their baseline warts showed reductions in all three viral markers.

3.4. Effects on keratinocyte and cell cycle markers

Since HPV infection is associated with hyperproliferation and inhibition of differentiation of keratinocytes, the effects of imiquimod on markers of proliferation and differentiation were assessed. Fig. 4 shows that imiquimod treatment leads to increased expression of the keratinocyte differentiation markers K10 at both week 6 and end of treatment and significant increases in filaggrin expression at week 6 ($P < 0.029$) when compared to vehicle-treated patients. In addition, trends toward decreases in mRNA for the proliferative markers c-myc and PCNA at week 6 were observed, with these effects being even more pronounced at end of treatment. The reduction in proliferative markers seen after treatment with imiquimod was accompanied by a trend toward increases in mRNA for the tumor suppressor genes pRb and p53. Results for the patient who cleared their warts while receiving placebo were similar to imiquimod treated patients in that levels of mRNA for PCNA were decreased at end of treatment, while levels of p53 at week 6 and levels of K10, filaggrin, pRb and p53 at end of treatment were elevated.

4. Discussion

Spontaneous regression of genital warts has been reported in 10–30% of patients and it is reported to be associated with an increase in leukocyte infiltration (Bishop et al., 1990; Coleman et al., 1994). In this study, one patient who received placebo cream experienced spontaneous clearance of baseline warts (Tyring et al., 1998). Evidence of a host response and virus reduction was seen in this patient, as measured by increases in mRNA for IFN-β, CD8, and CD29 and decreases in mRNA for CD1a, E7, L1, PCNA, and HPV DNA (Tyring et al., 1998), which may explain why statistically significant changes in some of these markers were not seen when comparing imiquimod- and placebo-treated patients in this report. The increase in mRNA for cell surface markers is not the best way to examine effects on cellular infiltrates; however, samples to be used for immunohistochemistry were thawed in transit and therefore were unavailable for analysis. Future studies employing immunohistochemical techniques will be used to confirm the results obtained in this study.

The increased incidence of genital warts and HPV infection in transplant patients and HIV patients argue strongly for the role of CMI in suppressing manifestations of this infection (Koutsky et al., 1988). The mechanism of both spontaneous and IFN induced regression of genital warts likely involves an effective host response to this viral infection (Bishop et al., 1990; Arany et al., 1994; Coleman et al., 1994; Arany and

Tyring, 1996a). Imiquimod may work via a similar mechanism to that reported in spontaneous clearance of warts since mRNA for the Th1 cytokine, IFN-γ, and mRNA for $CD4^+$ Th cells which are important for a CMI response, were elevated in the imiquimod-treated patients. Studies have also demonstrated the importance of the Th1 cytokine, IL-2 in wart clearance induced by IFN therapy (Arany and Tyring, 1996a). A significant correlation between mRNA for IL-2 and IFN-γ at week 6 ($P < 0.0076$), suggests that both cytokines are secreted at similar times. Induction of IFN-γ by imiquimod is probably an indirect effect that is mediated through cytokines such as IL-12 and IFN-α that are induced by the drug. In vitro studies have shown that antibodies to IFN-α and IL-12 abrogate the induction of IFN-γ by the immune response modifiers (Wagner et al., 1999).

Another effector cell in the CMI response is the $CD8^+$ CTL. Although studies involving spontaneous regression and regression induced by IFN do not stress the importance of $CD8^+$ T-cells, increases in these cells have been observed (Coleman et al., 1994; Arany and Tyring, 1996b). Thus, acquired immunity, specifically the cell mediated arm, seems to play an important role in immune mediated wart regression induced spontaneously, by IFN or by imiquimod; and the effects on CMI are likely a central mechanism for the action of the drug.

One of the consequences of increased CMI would not only be elimination or control of infection after completion of therapy, but also long-term protection against recurrence or reinfection. Clinical studies with imiquimod demonstrate good clearance rates and a low recurrence rate (10–13%) during 12 weeks of follow-up (Beutner et al., 1998; Edwards et al., 1998). The clinical data is supported by animal studies where imiquimod has led to long-term acquired immunity in both the guinea pig recurrent herpes simplex virus and the murine tumor models (Sidky et al., 1992; Tomai et al., 1997; Slade et al., 1998).

The LC is an important immunocompetent cell in the skin that is associated with presentation of antigen. It was somewhat surprising to see trends toward a decrease in CD1a mRNA expression in the imiquimod-treated patients and the patient whose warts spontaneously cleared. This observation has not been reported in studies evaluating patients whose warts spontaneously cleared or whose warts responded to prior IFN treatment (Coleman et al., 1994; Arany and Tyring, 1996b). The reduction in CD1a mRNA is not fully understood; however, studies in mice have recently demonstrated increased migration of LC following topical application of imiquimod to the skin (manuscript in preparation).

The previously reported data demonstrated that clearance of warts was associated with significant decreases in HPV DNA for types 6 and 11 and viral mRNA for L1 and E7 (Tyring et al., 1998). The reduction in L1 mRNA occurred prior to the reductions seen in HPV DNA and E7 mRNA, suggesting that initially imiquimod has a greater effect on more differentiated and superficial cells expressing L1 mRNA and containing replicating virus. This effect may be occurring closer to the skin surface in cells where the major capsid protein, L1 is being expressed. We have demonstrated that IFN is more effective in patients who express higher L1 levels (Arany et al., 1995a,b). The trend toward reduced E7 mRNA also appears later in therapy with imiquimod. Because HPV DNA levels are decreased along with L1 and E7 mRNA expression, it is possible that virus-infected cells are being eliminated. Reduction in HPV DNA correlated significantly with increases in mRNA expression for TNF-α ($P < 0.0026$), IL-12 p40 ($P < 0.012$), IFN-γ ($P < 0.048$), and CD4 ($P < 0.0028$), supporting a role for host response in elimination of HPV-infected cells.

The significant reduction in HPV DNA and in mRNA for L1 seen with drug suggests elimination of infected cells and a return to normal epithelium. This hypothesis is supported by an increase in mRNA for markers of more differentiated keratinocytes. In addition, proliferative markers such as c-myc and PCNA tended to decline following imiquimod treatment. Studies have shown that p53 mRNA levels are diminished in condylomas when compared to normal skin, while levels of pRb are actually increased (Arany et al., 1994). IFN treatment of condyloma leads to an even higher increase in pRb mRNA (Arany et al., 1994). Similar results were seen with imiquimod,

in that mRNA for p53 and pRb, tended to increase following treatment.

The goals of sexually transmitted disease therapy are the eradication of infection, elimination of symptoms, prevention of long-term sequelae, and interruption of transmission. The activation of IFN and CMI by imiquimod may lead to a significant reduction in viral load and elimination of clinically visible warts. This study supports the concept that imiquimod offers patients with anogenital warts a novel therapy that is self-applied and is safe and effective at treating both the symptoms and the underlying disease.

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