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Cytokine Induction in Hairless Mouse and Rat Skin After Topical Application of the Immune Response Modifiers Imiquimod and S-28463

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ALDARA (imiquimod cream 5%) recently became available for the treatment of genital and perianal warts; however, the topical mechanism of action of imiquimod is not fully understood. Imiquimod, and its analogs R-842, S-27609, and S-28463, are potent anti-viral and anti-tumor agents in animal models. Much of the biologic activity of these compounds can be attributed to the induction of cytokines, including interferon-α, tumor necrosis factor-α, interleukins-1, -6, -8, and others. This study was performed to characterize the response of mice and rats to topical application of imiquimod and S-28463 and also to evaluate these agents in cultures of murine and human skin cells. Topical administration of imiquimod or S-28463 to the flanks of hairless mice and rats leads to increases in local concentrations of interferon and tumor necrosis factor in the skin. The concentrations of interferon and tumor necrosis factor were higher at the site of drug application than in skin from the contralateral flank or skin from untreated animals. Interferon-α mRNA levels were also elevated in the skin of mice after topical application of either imiquimod or S-28463. *In vitro*, both imiquimod and S-28463 induced increases in interferon and tumor necrosis factor in cultures of cells isolated from hairless mouse skin. Imiquimod also increased interleukin-8 concentrations in human keratinocyte and fibroblast cultures, whereas S-28463 induced increases in tumor necrosis factor in fibroblast cultures. These results demonstrate that imiquimod and S-28463 stimulate production of cytokines in the skin after topical application, which may play a major role in its activity in genital wart patients. ***Key words: immunomodulator/interferon/interleukin/tumor necrosis factor. J Invest Dermatol 110:734–739, 1998***

Recently, ALDARA (imiquimod, R-837, or S-26308) 5% became available in the U.S.A. for the treatment of external genital and perianal warts. Imiquimod represents the first in a class of immune response modifiers that is patient applied. Clinical studies have demonstrated that imiquimod was significantly more effective than vehicle (placebo) in clearing genital/perianal warts. In addition, patients whose target warts cleared tended to remain clear during post-treatment follow-up periods of up to 12 wk.

The mechanism of action of imiquimod in genital wart patients is unknown; however, animal studies have shown that imiquimod and other members of the imidazoquinoline family (R-842, S-27609, and S-28463) are potent anti-viral and anti-tumor agents (Kende *et al*, 1988; Harrison *et al*, 1988, 1991, 1994; Chen *et al*, 1988; Bernstein and Harrison, 1989; Sidky *et al*, 1992; Tomai *et al*, 1995). These compounds possess no direct anti-viral or anti-tumor effects; rather, their activity is mediated in part through the induction of cytokines, in particular interferon (IFN)-α as demonstrated using IFN-α neutralizing antibodies (Kende *et al*, 1988; Sidky *et al*, 1992). IFN and other cytokines have been induced by imiquimod and S-28463 in several species including mice, rats, guinea pigs, monkeys, and humans following oral administration (Witt *et al*, 1993; Reiter *et al*, 1994; Tomai *et al*, 1995; Miller *et al*, 1995).

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In vitro studies have also shown the importance of cells from the immune system in the production of these cytokines. Human peripheral blood mononuclear cell (PBMC) cultures produce IFN-α, tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, and several other cytokines in response to the imidazoquinolines (Weeks and Gibson, 1993; Tomai *et al*, 1995; Gibson *et al*, 1995; Megyeri *et al*, 1995; Testerman *et al*, 1995). In particular, monocyte/macrophages are largely responsible for the cytokines produced in response to these drugs (Gibson *et al*, 1995; Megyeri *et al*, 1995) and the elevations in cytokines involve induction of cytokine mRNA and subsequent protein synthesis (Megyeri *et al*, 1995; Testerman *et al*, 1995).

The skin has recently been recognized as an important source of cytokines with several cells being able to secrete cytokines (Sauder, 1990; McKenzie and Sauder, 1990; VanDamme and Opdenakker, 1990; Katz, 1993; Schroder, 1995). Keratinocytes and dermal fibroblasts are capable of producing IFN-β, IL-1, IL-6, IL-8, IL-10, and the colony stimulating factors when exposed to UVB light, mitogens, lipopolysaccharide (LPS), or other cytokines (VanDamme *et al*, 1989; Sauder, 1990; McKenzie and Sauder, 1990; VanDamme and Opdenakker, 1990; Chodakewitz *et al*, 1990; Partridge *et al*, 1991; Katz, 1993; Swope *et al*, 1994). Finally, Langerhans cells found in the epidermis that function as the main antigen presenting cell in the skin, are also capable of secreting IL-1, IL-6, and TNF-α (Schreiber *et al*, 1992). These results suggest that cells found in the skin can produce cytokines if stimulated appropriately.

Imiquimod and S-28463 have been used in an attempt to stimulate keratinocytes, which are the major cell type found in the epidermis. Studies have shown that neither imiquimod nor S-28463 were cytotoxic

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in these cultures when tested at concentrations up to 100 μg per ml; however, cells from the human epidermal keratinocyte cell line, COLO-16, and normal human keratinocytes produce increased levels of IL-6 and IL-8 mRNA upon stimulation with imiquimod (Kono *et al*, 1994). S-28463 also induced IFN-α, TNF-α, and IL-8 mRNA as well as TNF and IL-8 protein in these cultures (Fujisawa *et al*, 1996). These studies indicate the potential of both imiquimod and S-28463 to activate cells found in the skin.

This study was undertaken to further elucidate the mechanism of action of imiquimod and S-28463 after topical application. Studies were performed in both hairless mice and hairless rats evaluating local cytokine production. In addition, *in vitro* culture systems were utilized to further define the cells in the skin that are responding to these molecules. These are the first studies to demonstrate local production of IFN and TNF following topical application of imiquimod or S-28463 to the skin of animals.

MATERIALS AND METHODS

Animals Female hairless SKH-1 mice (6–8 wk of age) and male hairless CD rats (6–8 wk of age) were purchased from Charles River Labs (Portage, MI). All protocols using mice and rats were reviewed by the 3M Institutional Animal Care and Use Committee.

Reagents The compounds imiquimod, 1-(2-methylpropyl)-1*H*-imidazo-[4,5-*c*]quinolin-4-amine, and S-28463, 4-amino-2-ethoxymethyl-α,α-dimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol, are proprietary molecules of 3M Pharmaceuticals (St. Paul, MN) and were used as either HCl salts or as free bases. The structures of these molecules have been described previously (Reiter *et al*, 1994; Tomai *et al*, 1995). For human and mouse cell culture studies, the HCl salts were dissolved in pyrogen-free water or culture medium and stored as stock solutions at 4°C for up to 4 mo. For dermal application, the free bases of imiquimod or S-28463 were dissolved in a cream or gel formulation, respectively. A stock solution of bacterial LPS from *Escherichia coli* 055:B5 (Sigma, St. Louis, MO) was dissolved at 1 mg per ml in pyrogen-free water and stored at 4°C until use.

Polyinosinic:polycytidylic acid (Pharmacia LKB, Milwaukee, WI) was prepared by dissolving the molecule in pyrogen-free water at 56°C for 30 min. The solution was then allowed to cool to room temperature before use.

Topical studies for local cytokine production A cream formulation of imiquimod or a gel formulation of S-28463 (10–100 μl) was applied topically over a 4 cm^2 area to the backs or flanks of mice that had been jacketed or of rats that had been collared to prevent ingestion of the drug. The drug was applied in 10–100 μl volumes and animals were sacrificed at 1, 2, 4, 6, or 24 h after treatment. Residual drug was removed using soap and water and then a 200–250 mg skin biopsy was removed from the site of application, snap frozen in liquid nitrogen, and used for either mRNA isolation or homogenized in 1 ml of RPMI medium containing 10% fetal calf serum (Sigma) for IFN and TNF protein determination.

mRNA isolation and reverse transcriptase polymerase chain reaction analysis mRNA was extracted from hairless mouse (SKH-1) skin biopsies using isolation kits purchased from Invitrogen (San Diego, CA) as previously described (Testerman *et al*, 1995). First-strand cDNA was synthesized from 0.1 μg of mRNA using random hexanucelotides (Boeringer Mannheim, Indianapolis, IN) and reverse transcriptase (Gibco BRL, Gaithersburg, MD), after which cDNA was amplified using specific primer sets for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), IFN-α, TNF, IL-1α, and IL-6 that were purchased from Clontech Laboratories (Palo Alto, CA). Sequences of these primer sets have been published previously (Testerman *et al*, 1995). The cDNA was amplified using Taq polymerase (Perkin Elmer, Norwalk, CT) and dNTP (Pharmacia Milwaukee, WI) in a Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer) for 30–35 cycles starting initially with a hot start (95°C for 2 min). The number of cycles was optimized for each primer pair and was shown to be in the linear range of amplification. Each cycle consisted of denaturing at 95°C for 15 s, annealing at 55°C for 15 s, and extending for 1 min at 72°C. Products were separated on 1.8% agarose gels and stained with ethidium bromide (Sigma). Phi X174 DNA digested with HAE III was run in each gel for molecular weight determinations. Polaroid photographs were taken of the gels while they were being exposed to UV light and band intensity was determined from these photographs using a Molecular Dynamics Densitometer (Sunnydale, CA). To ensure that intensities were in the linear range, dilutions of the sample were also performed. Areas for cytokine specific bands were normalized to the G3PDH bands and then multiplied by 100. The data are presented as the normalized mRNA expression for IFN-α.

Isolation of mouse skin cells Dorsal skin (200 mg) was obtained from 3–5 female SKH-1 hairless 6–8 wk old mice. The skin was incubated at 37°C with 100 U collagenase D per ml dissolved in medium for 1 h and then minced. Fragments were then incubated for an additional hour with 400 U collagenase D per ml, pressed through a sterile 80 mesh screen with a sterile glass pestle, and finally rinsed with 100 U collagenase D per ml to obtain a single cell suspension. The cell suspension was washed twice with phosphate buffered saline (Celox, Minneapolis, MN) and suspended at 1.5×10^6 cells per ml in RPMI medium with 10% fetal calf serum, 1% penicillin/streptomycin, and 5×10^{-5} M 2-mercaptoethanol (RPMI complete). Cells were incubated with various stimuli for 24 h at 37°C.

Cell lines Normal human epidermal keratinocytes, normal human dermal fibroblasts, and normal human epidermal melanocytes were purchased from Clonetics (San Diego, CA). Keratinocytes and melanocytes were cultured in modified MCDB 153 medium containing epidermal growth factor, antibiotics, insulin, and bovine pituitary extract. Fibroblasts were cultured in MCDB 2020 medium containing basic fibroblast growth factor, insulin, and antibiotics.

Cell culture for cytokine induction Keratinocytes, fibroblasts, and melanocyte cultures were grown to 90% confluence in 6 well tissue culture plates (Falcon, Lincoln Park, NJ). Fresh medium was added and cells were stimulated with imiquimod, S-28463, or Poly I:C for 24 h at 37°C + 5% CO_2. After incubation, supernatants were collected, filter sterilized, and stored at –20°C until cytokine analysis could be performed. Mouse skin cell cultures (1.5×10^6 cells per ml) were incubated in RPMI complete medium for 24 h following stimulation with imiquimod, S-28463, LPS, or Poly I:C. All cell-free supernatants were collected and stored for cytokine analysis as described below.

Cytokine analysis Mouse IFN was measured by bioassay using mouse L929 cells [American Type Culture Collection (ATCC), Rockville, MD] infected with encephalomyocarditis virus (ATCC) (Reiter *et al*, 1994). Human IFN was measured by bioassay using encephalomyocarditis virus infection of A549 (ATCC) target cells as described previously (Gibson *et al*, 1995). All other cytokines were measured by enzyme linked immunosorbent assay (ELISA). Mouse TNF ELISA kits were purchased from Genzyme (Cambridge, MA). Mouse IL-6, rat TNF, human TNF, and human IL-6 ELISA kits were purchased from Biosource (Camarillo, CA). The human IL-1α and IL-8 ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Statistical analysis Data were analyzed by a Student's t test. Results were considered significant only if $p < 0.05$. Both paired and unpaired tests were performed.

RESULTS

Cytokine concentrations in mouse and rat skin after topical application of imiquimod or S-28463 The first set of studies evaluated IFN and TNF concentrations in the skin after topical administration of 30 μl of imiquimod cream (5%). Results in **Fig 1(*a*)** show that mean concentrations of IFN (12 U per 20 mg tissue) and TNF (52 pg per 20 mg tissue) are low in the skin of untreated mice. One hour after treatment with imiquimod cream, significant induction of TNF-α is seen at the application site when compared with either skin from untreated animals or skin from the contralateral flank of the animal receiving the drug. IFN levels at the drug application site were also significantly elevated when compared with levels seen from the contralateral flank that did not receive the drug. At 2 h, both IFN and TNF concentrations are elevated significantly at the application site. Results from skin taken 4 h after application indicated significant increases in IFN and TNF at both sites (data not shown). Application of vehicle cream showed no significant induction of IFN or TNF when compared to untreated mice.

Results using topical application of the 1% imiquimod cream are presented in **Fig 1(*b*)**. Significant increases in IFN and TNF were seen at the application site 1 h after treatment. Low levels of IFN and TNF were again seen in skin from untreated animals. At 2 h after treatment, mean concentrations of IFN and TNF were significantly higher than those seen in the contralateral flank as well as those seen in skin from untreated mice.

S-28463 gel was tested at the 0.01%, 0.1%, and 1% (wt/vol) strengths for induction of IFN and TNF in the skin of hairless mice (**Table I**). Concentrations of TNF and IFN in skin from untreated mice were similar to those described above. The 0.01% gel formulation showed only low concentrations of IFN and TNF at the application site.

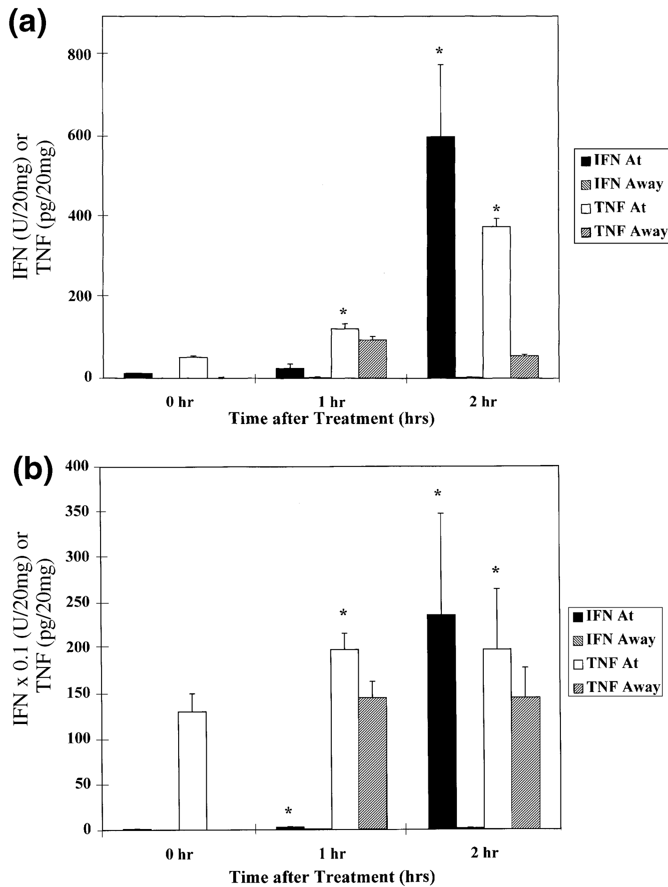

Figure 1. Local cytokine levels in the skin of hairless mice following topical imiquimod application. 5% Imiquimod cream (*a*) in a 30 μl volume or 1% imiquimod cream (*b*) in a 10 μl volume were applied to the backs of female hairless mice over a 2 cm^2 area. At various times after dosing, mice were sacrificed and a 200 mg skin biopsy was removed at the site of drug treatment (At) and from a site distant (Away). TNF was measured by ELISA and IFN was determined by bioassay. Results are presented as the mean of three animals per point ± SEM and are expressed as U per 20 mg biopsy for IFN or pg per 20 mg biopsy for TNF. *Significant difference ($p < 0.05$) when compared with the untreated control and when compared with the "away" site.

Significant induction of IFN by the 0.1% strength of S-28463 gel was seen at the application site 2 and 4 h after dosing. TNF concentrations were also elevated slightly at all three time points, with the elevation at 1 h being statistically significant. High concentrations of IFN were observed after administration of the 1% strength of S-28463 gel; however, no difference was seen when comparing concentrations at the site of application and those at the site not receiving the drug. IFN levels in skin taken from the application site and skin taken from the contralateral flank were significantly increased when compared with skin from untreated mice. TNF concentrations at the application site were significantly higher than those seen in the contralateral flank. Thus, imiquimod and S-28463, at the strengths tested, were capable of inducing local cytokine production in the skin following topical application.

Further studies were performed in hairless rats to determine if the topical effects of these drugs were limited to mice and to determine if these drugs were effective topically in animals with thicker skin. In rats, only TNF was evaluated. Results in **Fig 2** demonstrate that the 5% imiquimod cream induced significant increases in TNF concentra-

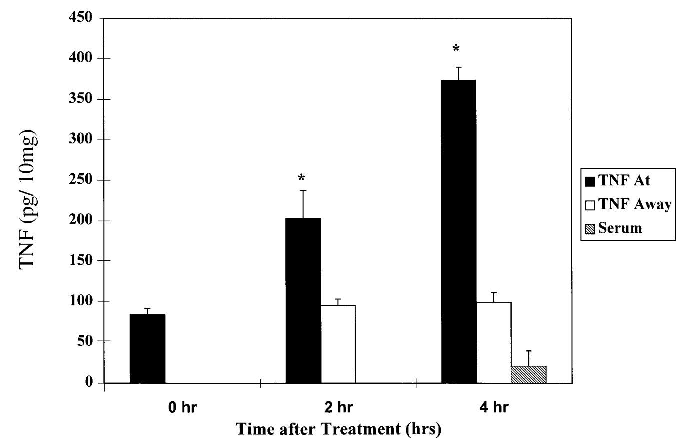

Figure 2. TNF induction in the skin of hairless rats by topical application of 5% imiquimod cream. Imiquimod 5% cream (100 μl) was applied to the right flanks of male rats over a 4 cm^2 area. At 2 and 4 h after dosing, rats were sacrificed and a 200 mg skin biopsy was removed at the site of drug treatment (At) and from a site on the other flank where drug was not applied (Away). TNF was measured by ELISA and results are presented in pg per 10 mg biopsy and represent the mean of six rats per treatment ± SEM. *Significant difference ($p < 0.05$) when compared with the site that did not receive drug and also with skin taken from untreated rats.

Table I. TNF and IFN induction in hairless mouse skin by topical S-28463 application[a]

		Cytokine concentration per 200 mg biopsy			
		TNF (pg)		IFN (U)	
Drug concentration	Time (h)	At	Away	At	Away
0.01%	0	125 ± 25	–	1 ± 0.3	–
	1	97 ± 27	193 ± 44	0	0
	2	185 ± 32	183 ± 24	0.9 ± 0.6	0
	4	192 ± 36	187 ± 19	1.5 ± 0.8	0
0.1%	0	110 ± 16	–	0	–
	1	146 ± 28[b,c]	113 ± 21	2.7 ± 1.1	1.6 ± 0.7
	2	289 ± 31[c]	202 ± 29[c]	239 ± 143[b,c]	1.3 ± 0.8
	4	330 ± 66[c]	259 ± 34[c]	248 ± 104[b,c]	54 ± 37[c]
1.0%	0	173 ± 35	–	2 ± 1	–
	1	861 ± 71[b,c]	375 ± 73[c]	60 ± 20[c]	56 ± 18[c]
	2	1210 ± 284[b,c]	865 ± 273[c]	1691 ± 343[c]	1569 ± 459[c]
	4	372 ± 154[b,c]	195 ± 100	729 ± 486[c]	498 ± 255[c]

[a]S-28463 gel (10 μl) was applied to the backs of female mice at 0.01, 0.1, and 1.0% drug concentrations. At various times after dosing, mice were sacrificed and a 200 mg skin biopsy was removed at the site of drug treatment (AT) and from a site distant (Away). TNF was measured by ELISA and IFN was determined by bioassay with results being presented in pg and U, respectively. Data are presented as the mean ± SEM of three mice per point assayed individually.

[b]Significant difference ($p < 0.05$) when compared with the site where the drug was not applied.

[c]Significant difference ($p < 0.05$) when compared with skin from untreated mice.

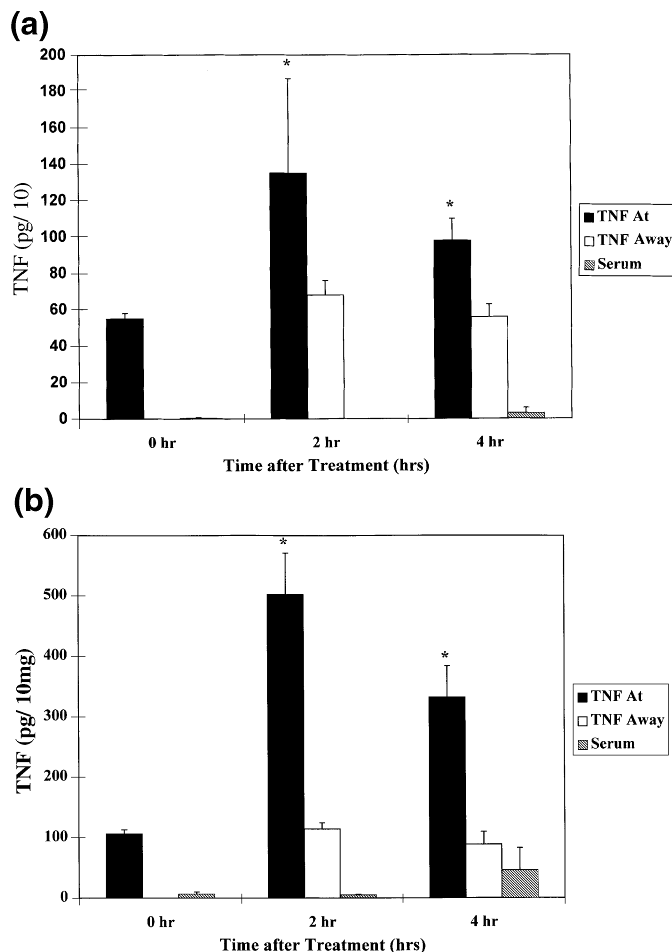

Figure 3. TNF induction in hairless rat skin by topical application of 0.05% and 0.25% S-28463 gel. S-28463 at 0.05% (*a*) or 0.25% (*b*) in a gel formulation (100 μl) was applied to the right flanks of male rats over a 4 cm^2 area. At 2 and 4 h after dosing, rats were sacrificed and a 200 mg skin biopsy was removed at the site of drug treatment (At) and from a site on the other flank where drug was not applied (Away). TNF was measured by ELISA with results being presented in pg per 10 mg biopsy and represent the mean of six rats per treatment ± SEM. *Significant difference ($p < 0.05$) when compared with the site that did not receive the drug and also compared with skin taken from untreated rats.

tions at the site of application when compared with skin from untreated rats and skin taken from the contralateral site. Peak concentrations of TNF were seen at 4 h. Serum concentrations of TNF were evaluated and no elevations were seen after drug application.

S-28463 gel at either the 0.05 or the 0.25% strength was also tested for local TNF induction in hairless rats. Results in **Fig 3** present the data for both the 0.05% (**Fig 3*a***) and the 0.25% (**Fig 3*b***) gel formulations of S-28463. Skin from animals receiving the 0.05% strength of S-28463 had significantly higher levels of TNF at the application site when compared with skin from either the contralateral flank or the untreated rats. These elevations were seen at both 2 and 4 h. Similar results were seen when applying the 0.25% gel strength. Significant elevations in TNF (2 and 4 h) were seen with peak concentrations at 2 h. No differences were seen between skin taken from untreated rats and skin taken from the untreated sites of animals that had been dosed. Finally, neither the 0.05 nor the 0.25% formulation of S-28463 increased TNF concentrations in the serum. These results demonstrate that topical application of either imiquimod or S-28463 to the skin of animals leads to increased cytokine production at the site of application.

Induction of IFN-α mRNA in mouse skin after topical drug treatment To determine whether the induction of IFN was through activation of local transcription or not, imiquimod cream 5% and S-

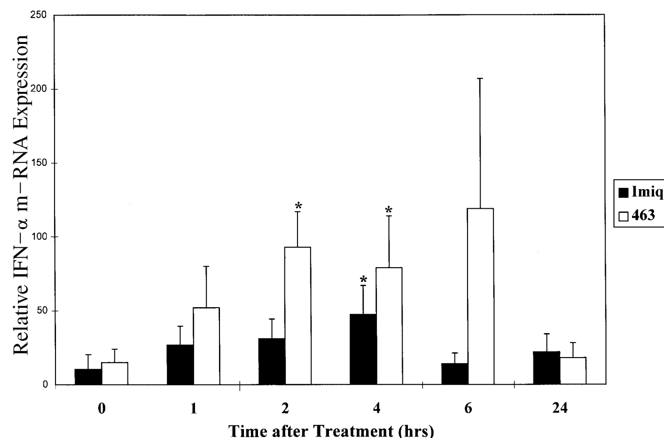

Figure 4. Induction of IFN-α mRNA in mouse skin after topical application of imiquimod 5% cream or S-28463 1% gel. 5% Imiquimod cream (100 μl) or 1% S-28463 gel (100 μl) was applied to the backs of female hairless mice over a 2 cm^2 area. At various times after dosing, mice were sacrificed, skin was removed, and mRNA was isolated and subjected to reverse transcriptase polymerase chain reaction. Products were separated by agarose gel electrophoresis and band intensity was quantitated by densitometry. IFN band intensity was normalized to the house keeping gene, G3PDH, to control for differences in starting amounts of mRNA, and multiplied by 100. Results are presented as the mean ± SEM of three individual experiments. *$p < 0.05$ when compared with the 0 h sample.

28463 gel 1% were applied to the skin of hairless mice and mRNA was semiquantitated by reverse transcriptase polymerase chain reaction. Application of imiquimod caused a significant increase in IFN-α mRNA at 4 h after administration when compared with untreated animals (**Fig 4**). The 1 and 2 h samples also showed an increase in mRNA for IFN-α, although this was not statistically significant. At 6 and 24 h, the mRNA levels for IFN-α were similar to control levels. mRNA levels for TNF were not significantly elevated in skin receiving topical imiquimod (data not shown).

S-28463 gel 1% also significantly increased IFN-α mRNA levels, compared with skin from untreated mice, at 2 and 4 h after a single application. In addition, there was an increase in mRNA at the 1 and 6 h time points; however, these were not statistically significant. IFN-α mRNA levels had returned to control levels in the samples taken 24 h after application. Induction of IFN-α mRNA was also noted using the 0.1% strength of S-28463 gel (data not shown). Cytokine specific mRNA for TNF was also evaluated after a single administration of S-28463 with no induction noted when compared with the placebo control. Thus, topical application of imiquimod or S-28463 leads to increased IFN-α mRNA levels that are responsible for the increase in protein.

Cytokine induction in isolated mouse skin cell preparations and human keratinocyte, fibroblast, and melanocyte cultures Because there are several cell types in the skin that are able to produce cytokines, it was important to evaluate *in vitro* the cells from skin that may be responsible for the cytokines produced after topical drug treatment. Results of cytokine induction in unseparated mouse skin cell preparations are presented in **Fig 5**. Control cultures receiving medium alone had relatively high concentrations of both IFN and TNF when compared with cultures of cells from either spleen or bone marrow. Imiquimod induced increased IFN levels above untreated control cultures at both 3 and 1 μg per ml but did not increase TNF levels at either concentration tested. S-28463 showed significant induction of both IFN and TNF at 1 μg per ml when compared with untreated control cultures. LPS induced a statistically significant increase in IFN and TNF concentrations. Poly I:C induced significant increases in IFN production without significantly increasing TNF production. These studies indicate that cells isolated from the skin are capable of secreting increased concentrations of both IFN and TNF in response to imiquimod or S-28463.

Several isolated human cell preparations including keratinocytes,

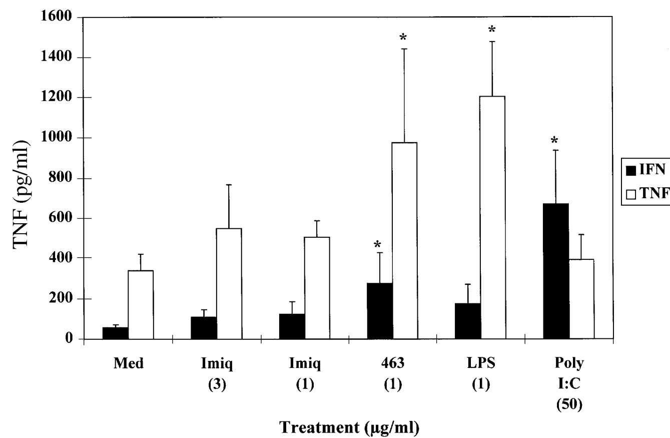

Figure 5. Cytokine induction in mouse skin cell preparations by imiquimod and S-28463. Mouse skin cells (1.5×10^6 per ml) were stimulated for 24 h with imiquimod (Imiq), S-28463 (463), LPS, or Poly I:C. Cell free supernatants were collected for cytokine analysis. The data are presented as the mean ± SEM from three to seven experiments. *Statistically significant difference ($p < 0.05$) when compared with the untreated control cultures.

fibroblasts, and melanocytes were evaluated for cytokine production in response to imiquimod and S-28463. These drugs were not cytotoxic for any of these cell types at concentrations up to 100 μg per ml. Imiquimod, but not S-28463, was effective at stimulating significantly increased production in IL-8 concentrations in keratinocyte cultures (2 pg per ml in untreated cultures *versus* 28 pg per ml in imiquimod treated cultures). IFN, IL-1, IL-6, and TNF were not increased by either imiquimod or S-28463. In fibroblast cultures, imiquimod was again able to significantly increase IL-8 concentrations 2-fold over background, whereas S-28463 significantly increased TNF concentrations (2–3-fold). No significant increases were seen in IFN, IL-1, or IL-8 concentrations in fibroblast cultures stimulated with either compound. Finally, neither imiquimod nor S-28463 caused any increase in IFN, IL-1, IL-6, IL-8, or TNF concentrations in melanocytes.

DISCUSSION

Although imiquimod cream 5% has recently become available for the treatment of external genital and perianal warts, the mechanism of action is not fully understood. Most of the mechanistic studies reported thus far have focused on systemic routes of application or *in vitro* studies using immunocompetent cell populations. This study focused on cytokine induction by imiquimod and the more potent analog S-28463 after topical application to skin. Results reported herein extend previous findings by demonstrating local production of cytokines in the skin following topical application. Local cytokine production by topical application of these agents was demonstrated in both mice and rats, indicating that this result is not species specific.

The kinetics of cytokine induction following topical application of these drugs occurs within hours. Similar kinetics for induction of IFN and TNF were seen between imiquimod and S-28463 when evaluated in mice. The kinetics are shifted slightly to the right when compared with kinetics seen after systemic dosing of mice with these compounds, which may be due to slightly slower absorption by the topical route (Miller *et al*, 1995). In rats, peak concentrations of TNF are seen slightly earlier for S-28463 gel when compared with imiquimod cream. One possible explanation is that S-28463 gel is able to penetrate rat skin more effectively. IFN was not evaluated in rat samples due to toxicity of the skin homogenates in the rat IFN bioassay. With regard to potency, S-28463 was active at strengths of 0.05% and above, whereas imiquimod was active at strengths only above 1%, indicating that S-28463 was at least 20-fold more potent in this topical model. Similar results have been observed using systemic administration of the drug as well as *in vitro* studies (Tomai *et al*, 1995). The kinetics of cytokine induction after topical application of the drug suggests that induction is rapid and is likely through activation of cells already present in the skin.

The induction of cytokines in the skin by imiquimod and S-28463 appears to be due to local stimulation. In both mice and rats, concentrations at the application site were almost always higher than those seen when skin was taken from the flank that did not receive the drug or from untreated animals. Also, in hairless rats there was no increase in serum concentrations of TNF following topical application of either drug. In mice treated with high strengths of S-28463 (1.0%), IFN and TNF were seen at both biopsy sites leading to the possibility that some of the drug may be reaching the systemic circulation. Alternatively, the cytokines seen at the site where the drug was not applied could be a result of locally produced cytokine entering the systemic circulation. Studies are in progress to define which hypothesis is correct. These results indicate that the topical application leads to local production of cytokines without generating a systemic response.

The induction of local cytokines by these drugs is probably due to increases in transcription, because increases in IFN-α mRNA were also seen in skin from drug treated animals. The kinetics of induction of IFN-α mRNA indicated increases as early as 1 h after dosing with levels returning to background by 6–24 h. The increases in mRNA for IFN-α correlated quite well with the kinetics for detection of the protein. These results again indicate that these drugs activate cells in the skin to produce IFN, specifically IFN-α.

The inability to detect increases in TNF mRNA was surprising; however, it may be that earlier sampling was necessary to detect increases in this mRNA. It is possible that the polymerase chain reaction technique used may not be able to detect subtle differences in mRNA levels or that the increases in TNF may be due to the release of stored protein, as is seen with mast cells (Gordon and Galli, 1991); however, this latter possibility seems unlikely because mRNA and protein synthesis are required for cytokine induction by imiquimod and S-28463 in other systems (Testerman *et al*, 1995).

Studies have shown that cells of the monocyte/macrophage lineage are capable of producing cytokines in response to the imidazoquinolines (Tomai *et al*, 1995; Gibson *et al*, 1995; Megyeri *et al*, 1995; Testerman *et al*, 1995). *In vitro* results using cells isolated from mouse skin correlate well with the results obtained *in vivo* following topical application of the drug. Studies using cultures of mouse skin cells showed increases in IFN and TNF in response to these drugs. One problem with this system was the high concentrations of IFN and TNF seen in the control cultures. This was most likely a result of the isolation procedure. S-28463, but not imiquimod, was effective at increasing production of TNF in these cultures. This is not surprising because imiquimod is fairly ineffective at inducing TNF in human PBMC (Testerman *et al*, 1995). These results indicate that cells in the skin are capable of secreting cytokines in response to either imiquimod or S-28463.

Previous studies by Sauder *et al* have shown increases in cytokine specific mRNA for human keratinocyte cultures with imiquimod or S-28463 (Kono *et al*, 1994; Fujisawa *et al*, 1996). Imiquimod only stimulated increases in IL-8 concentrations in keratinocyte cultures, whereas S-28463 was ineffective at stimulating any of the cytokines evaluated. There may be several explanations as to why the results in this study did not correlate with the results obtained by Sauder *et al*. It is possible that the mRNA for IL-6 and IL-8 was not translated into protein, or that the ELISA used to measure IL-6 and IL-8 concentrations were not sensitive enough to detect the small differences in cytokine concentrations that may be present. Finally, it may be that the cytokines were quickly bound to cytokine receptors or that receptors for these cytokines are induced by these drugs. The inability to detect induction of cytokines by imiquimod or S-28463 is not due to an inability of keratinocytes to make cytokines, because Poly I:C was very effective at stimulating these cells to produce cytokines.

Results from this study also show that fibroblasts are poor responders to both imiquimod and S-28463. Increases in IL-8 were seen following imiquimod stimulation and increases in TNF were seen following S-28463 stimulation. These elevations were minimal when compared with Poly I:C, which was very effective at inducing IFN, IL-6, and IL-8 in these cultures. Published results using Poly I:C have also shown induction of IFN-β, IL-6, and IL-8 in fibroblast cultures (VanDamme *et al*, 1989; VanDamme and Opdenakker, 1990). These results suggest

that, if stimulated properly, keratinocytes and fibroblasts can secrete high levels of IFN and several proinflammatory cytokines.

Other cell types found in the skin, such as Langerhans cells and dermal macrophages, are known to secrete cytokines. Langerhans cells, the major antigen presenting cells in the epidermis, as well as the dermal macrophage, may be major contributors to the response seen after topical application of these drugs. Studies using enriched dendritic populations suggest that these cells do respond by producing cytokines to imiquimod and S-28463 (personal observation). These cells may play a prominent role in the initial response to imiquimod and S-28463 following topical application.

In conclusion, the results described in this study demonstrate that both imiquimod and the more potent analog S-28463 are capable of inducing local production of IFN and TNF following topical application. Local induction of these cytokines in the skin may play an important role in the efficacy of imiquimod in the treatment of external genital and perianal warts, and may suggest other possible topical disease targets that may benefit from this therapy.

REFERENCES

Bernstein DI, Harrison CJ: Effects of the immunomodulating agent R-837 on acute and latent herpes simplex virus type 2 infections. *Antimicrob Agents Chemother* 33:1511–1515, 1989

Chen M, Griffith BP, Lucia HL, Hsuing GD: Efficacy of S-26308 against guinea pig cytomegalovirus. *Antimicrob Agents Chemother* 32:678–683, 1988

Chodakewitz JA, Lacy J, Edwards SE, Birchall N, Coleman DL: Macrophage colony-stimulating factor production by murine and human keratinocytes. *J Immunol* 144:2190–2196, 1990

Fujisawa H, Shivji GM, Kondo S, Wang B, Tomai MA, Miller RL, Sauder DN: Effect of a novel topical immunomodulator, S-28463, on keratinocyte cytokine gene expression and production. *J Interferon Cytokine Res* 16:555–559, 1996

Gibson SJ, Imbertson LM, Wagner TL, Testerman TL, Reiter MJ, Miller RL, Tomai MA: Cellular requirements for cytokine production in response to the immunomodulators imiquimod and S-27609. *J Interferon Res* 15:537–545, 1995

Gordon JR, Galli SJ: Release of both preformed and newly synthesized tumor necrosis factor α (TNF-α) /cachectin by mouse mast cells stimulated via the $Fc_{\epsilon}RI$. A mechanism for the sustained action of mast cell-derived TNF-α during IgE-dependent biological responses. *J Exp Med* 174:103, 1991

Harrison CJ, Jenski L, Voychehovski T, Bernstein DI: Modification of immunological responses and clinical disease during topical R-837 treatment of genital HSV-2 infection. *Antiviral Res* 10:209–224, 1988

Harrison CJ, Stanberry LR, Bernstein DI: Effects of cytokines and. R-837, a cytokine inducer, on UV-irradiation augmented recurrent genital herpes in guinea pigs. *Antiviral Res* 15:315–322, 1991

Harrison CJ, Miller RL, Bernstein DI: Post therapy suppression of genital (HSV) recurrences and enhancement of HSV-specific T-cell memory by imiquimod in guinea pigs. *Antimicrob Agents Chemother* 38:2059–2064, 1994

Katz S: The skin as an immunological organ: allergic contact dermatitis as a paradigm. *J Invest Dermatol* 20:593–603, 1993

Kende M, Lupton HW, Canonico PG: Treatment of experimental viral infections with immunomodulators. *Adv Biosciences* 68:51–63, 1988

Kono MD, Kondo S, Shivji GM, Venner TJ, Tomai MA, McKenzie RC, Sauder DN: Effects of the immunomodulator R-837 on cytokine gene expression in the human epidermal carcinoma cell line COLO-16. *Lymphokine Cytokine Res* 13:71–76, 1994

McKenzie RC, Sauder DN: The role of keratinocyte cytokines in inflammation and immunity. *J Invest Dermatol* 95:105S–107S, 1990

Megyeri K, Au W, Rosztoczy I, Raj NB, Miller RL, Tomai MA, Pitha PM: Stimulation of interferon and cytokine gene expression by imiquimod and stimulation by Sendai virus utilize similar signal transduction pathways. *Mol Cell Bio* 15:2207–2218, 1995

Miller RL, Birmachu W, Gerster JF, *et al*: Imiquimod: cytokine induction and antiviral activity. *Antiviral News* 3:111–113, 1995

Partridge M, Chartry D, Turner M, Feldmann M: Production of interleukin-1 and interleukin-6 by human keratinocytes and squamous cell carcinoma cell lines. *J Invest Dermatol* 96:771–776, 1991

Reiter MJ, Testerman TL, Miller RL, Weeks CE, Tomai MA: Cytokine induction in mice by the immunomodulator imiquimod. *J Leuk Biol* 55:234–240, 1994

Sauder DN: The role of epidermal cytokines in inflammatory diseases. *J Invest Dermatol* 95:27S–28S, 1990

Schreiber S, Kilgus O, Payer E, Kutil R, Elbe A, Mueller C, Stingl G: Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. *J Immunol* 149:3525–3534, 1992

Schroder JM: Cytokine networks in the skin. *J Invest Dermatol* 1:20S–24S, 1995

Sidky YA, Borden EC, Weeks CE, Reiter MJ, Hatcher JF, Bryan GT: Inhibition of murine tumor growth by an interferon inducing imidazoquinoline. *Cancer Res* 52:3528–3533, 1992

Swope VB, Sauder DN, McKenzie RC, *et al*: Synthesis of interleukin-1α and β by normal human melanocytes. *J Invest Dermatol* 102:749–753, 1994

Testerman TL, Imbertson LM, Reiter MJ, Miller RL, Wagner TL, Tomai MA: Cytokine induction by the immunomodulators imiquimod and S-27609. *J Leuk Biol* 58:365–372, 1995

Tomai MA, Gibson SJ, Imbertson LM, *et al*: Immunomodulating and antiviral activities of the imidazoquinoline S-28463. *Antiviral Res* 28:253–264, 1995

VanDamme JV, Opdenakker G: Interaction of interferons with skin reactive cytokines: from interleukin-1 to interleukin-8. *J Invest Dermatol* 95:90S–93S, 1990

VanDamme J, Schaafsma MR, Fibbe WE, Falkenburg JHF: Simultaneous production of Interleukin-6, interferon -β and colony-stimulating activity by fibroblasts after viral and bacterial infection. *Eur J Immunol* 19:1189–1194, 1989

Weeks CE, Gibson SJ: Induction of interferon and other cytokines by imiquimod and its hydroxylated metabolite R-842 in human blood cells in vitro. *J Interferon Res* 14:93–97, 1993

Witt KA, Ritch PS, Reding D, McAuliffe Westrick L, Grossberg SE, Borden EC: Phase I trial of an oral immunomodulator and interferon inducer in cancer patients. *Cancer Res* 53:5176–5280, 1993