Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential

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Summary

A central development of the past decade has been in our understanding of the interactions between, and interdependence of, the innate and adaptive immune responses. Innate immunity recognizes 'danger' signals and activates adaptive immunity in a targeted, appropriate and effective response. Dendritic cells and macrophages have a central role in this process, and pharmacological agents that modulate the functions of these cells could have therapeutic value. The imidazoquinolone compounds, of which imiquimod, formulated as Aldara^{TM}, is the best characterized to date, are such molecules. Imiquimod and its homologues act by activating macrophages and other cells via binding to cell surface receptors, such as Toll receptor 7, thereby inducing secretion of pro-inflammatory cytokines, predominantly interferon (IFN)-α, tumour necrosis factor (TNF)-α and interleukin (IL)-12. This locally generated cytokine milieu biases towards a Th1 cell mediated immune response with the generation of cytotoxic effectors, and this has been exploited clinically in the treatment of viral infections (human papillomavirus, herpes simplex virus, molluscum contagiosum) and nonmelanoma skin cancer. Imiquimod has been shown to be significantly more effective than placebo in clearing genital warts, and mechanism of action studies indicate that this is related to the ability to generate proinflammatory cytokines and a Th1 response. Intra-epithelial neoplasms of cutaneous and mucosal surfaces are associated with human papillomavirus infection and there is some evidence that immune response modifiers may have therapeutic value for these lesions. Topical immunotherapy with immunomodulators shows potential for effective and patient-friendly treatment of cutaneous viral infections. These compounds also have adjuvant properties that could significantly enhance conventional vaccine strategies.

Introduction

A central development of the past decade has been in our understanding of the interactions between, and interdependence of, innate and adaptive immunity.[1] The innate immune system (mononuclear phagocytes, complement and epithelial barriers) is evolutionarily ancient and present in some form in all multicellular organisms. Innate immunity is hard-wired using proteins encoded in the germline to identify potentially noxious elements. Adaptive immunity, in contrast, is found only in vertebrates, is almost infinitely flexible and is based on lymphocyte receptors generated by somatic gene rearrangement during ontogenesis. Using the products of the Recombination activating genes (RAG)1 and RAG2 genes, B and T lymphocytes rearrange the variable (V), diversity (D) and joining (J) genes of their immunoglobulin (Ig) or T-cell receptors to generate in excess of 10^{11} clones of B and T lymphocytes expressing distinct antigen-specific receptors. The specificity of these receptors is not predetermined, and neither is the response that might be induced in the

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lymphocytes after the ligation of the receptor by antigen. It is now clear that the innate immune response generates the signals that identify the nature of the antigen and the type of effector response to be induced.

The germline-encoded receptors of the innate immune system recognize conserved molecular targets that are essential products of microbial physiology central to microbial survival. These invariant products, known as pathogen associated molecular patterns (PAMPs), are not specific to a particular pathogen but are shared by groups of pathogens. They include molecules such as lipopolysaccharide, peptidoglycans, double-stranded RNA and mannans. The PAMPs are recognized by receptors of the innate immune system called pathogen recognition receptors (PRRs). The PAMPs are seen by the innate immune system as a barcode for the microbes that bear them, and this leads to the induction of an adaptive immune response. The PRRs expressed on the cell surface are toll-like receptors (TLRs). Activation of the TLRs of dendritic cells results in the expression of costimulatory molecules necessary for the activation of naïve T cells with a T-cell receptor (TCR) specific for the antigenic peptides derived from the microbes and expressed as a peptide/MHC complex on the dendritic cell (DC). Antigen-specific T cells are activated and an effective, appropriate, targeted adaptive immune response is initiated.

Adaptive immune responses are divided into type 1 (cell mediated, Th1) and type 2 (humoral, Th2), and naïve T cells differentiate down the Th1 or Th2 path in response to signals from the innate immune system in which DCs and macrophages are key players. Activation of tissue macrophages via certain TLRs or ligation of other receptors results in the secretion of IL-12 and TNF-α which bias activated naïve T cells to the Th1 path. Antigen-activated T cells express the CD40 ligand, which can bind to CD40 on macrophages inducing secretion of IL-12 and TNF, which then synergize with IL-2 from activated T cells inducing further IFN-γ from T cells and natural killer (NK) cells, in turn inducing more macrophage secretion of IL-12. Thus a positive autocrine feedback loop is generated which drives macrophage activation, T-cell generation and function and amplifies NK cell activity.

Imiquimod: a modulator of innate immunity

The key role of the innate immune system in initiating effective, appropriate and targeted adaptive immune responses has profound implications for immunotherapies. The central role of DCs and macrophages in the induction and regulation of Th1 responses implies that pharmacological agents that modulate the functions of these cells might have therapeutic value. Such agents are the imidazoquinolone compounds (Fig. 1) of which imiquimod, a novel synthetic molecule developed by 3M Pharmaceuticals (St Paul, MN), is the best known to date. This compound, formulated as Aldara™, has been evaluated as a topical therapy for genital warts in phase III studies where it has shown safety and efficacy.[2] It has also shown efficacy and safety in phase I/II trials as a topical therapy for basal cell carcinoma.[3] The evidence reviewed below indicates that imiquimod and its homologues modulate innate immune responses by activating DCs, macrophages and other cells via Toll receptor 7 (TLR-7) binding to induce the synthesis of IFN-α and pro-inflammatory Th1 cytokines (Table 1).

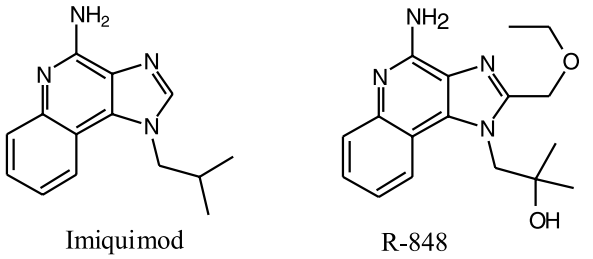

Figure 1 Chemical structure of imiquimod and resimiquimod (R-848). Imiquimod superficially resembles a nucleoside analogue but lacks the fourth nitrogen that is present in a purine. It contains the imidazo-quinoline group as a third ring and the isobutyl group in the 1 position, which would be occupied by ribose or deoxyribose in a nucleoside.

Table 1 Cytokines and chemokines induced by imidazoquinolines.

Interferons	*IFN- α, IFN-β
Interleukins	IL-1, IL-6, IL-8, IL-10, *IL-12
Tumour necrosis factor-α	*TNF-α
Interleukin 1 receptor antagonist	IL-1RA
Granulocyte colony stimulating factor	G-CSF
Granulocyte-macrophage colony stimulating factor	GM-CSF
Macrophage inflammatory protein	MIP-1α and MIP-1β
Macrophage chemotactic protein	MCP

*Cytokines induced consistently in imiquimod-treated PBMCs.

Mechanism of action: evidence from preclinical studies using animal models

Imiquimod was first shown to be a potent inducer of cytokines using the guinea pig model of herpes simplex virus (HSV) infection.[4] Administration of the drug intravaginally completely protected against primary

disease and reduced recurrent disease significantly, producing a dramatic decline in virus shedding within 24 h.[5] These effects correlated with the IFN titres induced in the treated animals.[4] Oral imiquimod induces high levels of circulating IFN-α in a range of species including rodents[6] and primates[7] with a minimal effective dose of 2–3 mg/kg. In mice, maximal IFN levels were induced 2 h post dosing with 30 mg/kg, but serum IFN was undetectable at 4 h post dose. Higher drug doses, 100 mg/kg, resulted in lower peak serum IFN but these persisted for 24 h or more. Interferons were not the only cytokines induced. IL-6 levels paralleled that of interferons, and TNF-α induction occurred 1 h post dosing with 30 mg/kg but was undetectable at 4 h.[8]

Mouse models have been used to analyse the response to topical imiquimod.[9] In these experiments, 5% imiquimod in a cream formulation was applied to the skin of hairless mice. Biopsies of drug-treated and vehicle-treated sites were taken at different time points post treatment and RT-PCR performed to detect mRNA for a range of cytokines including IFN-α, IFN-β, TNF-α, IL-1α, IL-1β, IL-6 and IL-12. IFN-α and TNF-α mRNAs were measurable at 1 and 2 h post treatment but only IFN-α was detectable at 4 h post treatment. Cytokine levels peaked at 2 h in the treated sites.

Imiquimod effects on dendritic cells and macrophages: *in vitro* studies

Peripheral blood mononuclear cells (PBMCs), when grown *in vitro* in the presence of imiquimod, are induced to release interferon.[10] The efficiency of interferon production is dose related with a threshold concentration for human PBMCs of 0.5 μg/mL (approximately 2 μM), making imiquimod a relatively efficient inducer of cytokines. The cell population responsible for the bulk of IFN-α release from human PBMCs *in vitro* is a member of the monocyte macrophage lineage.[10] Imiquimod and its analogue S-27609 were evaluated for their ability to induce a range of cytokines in human PBMCs.[11] Both compounds induced IFN-α, TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1α and the IL-1 receptor. Cytokine induction was evident within 2 h of treatment and maximal concentrations were measured approximately 8 h after the addition of the drug. The first cytokine detected was IFN-α; maximal concentrations were achieved after 8 h and remained reasonably constant over 24 h. TNF, IL-1 and IL-6 concentrations also peaked at 8 h but maximal concentrations were significantly lower than those achieved for IFN and IL-8.

Cultured and wound keratinocytes are potent sources of pro-inflammatory cytokines and imiquimod treatment of neonatal foreskin keratinocytes for 24 h resulted in an up-regulation of mRNA expression for IFN-α, IL-6 and IL-8, although only small amounts of protein for IL-8 were detectable.[12] Imiquimod treatment of T cells has no effect on T-cell proliferation, IL-2 production or IL-2 receptor expression. However, treatment of human PBMCs and mouse spleen cultures results in the production of IFN-γ, and this can be inhibited by treatment with anti-IL-12 antibody. Th2 cytokines such as IL-5 are down-regulated in imiquimod-treated PBMCs.[13]

Antiviral effects: *in vivo* studies

Imiquimod has not been shown to exert a direct antiviral effect *in vitro* or *in vivo* but indirect antiviral activity has been demonstrated.[14] *In vivo* antiviral activity was first demonstrated in the guinea pig model of HSV-2 infection.[4,5] In this model, early treatment with intravaginal imiquimod reduced HSV latency but latency once established could not be reversed by imiquimod. The antiviral effects in this model were related to the induction of IFN-α, the IFN inducible enzyme 2′5′ oligoadenylate synthetase and augmentation of T-cell responses.[15] The adjuvant effect for immunotherapy of recurrent HSV infection has been demonstrated recently in a randomized placebo-controlled trial of imiquimod alone or with a glycoprotein vaccine. Both regimes reduced recurrence but imiquimod + vaccine extended the duration and protection against recurrence compared to drug alone.[16] Ongoing clinical trials with resiquimod, a homologue of imiquimod, are assessing the effects on recurrences of genital herpes in men. Early reports show a significant reduction in mean time to recurrence in resiquimod-treated patients compared to 69 days in the control group.

Receptor ligand interactions

Studies with the ^{14}C-labelled imiquimod analogue S26704 have shown that the metabolite binds to both high- and low-affinity sites on the plasma membrane of a mouse macrophage cell line. This binding is saturable and competitive and suggests that imiquimod and/or its metabolites bind to a cell surface receptor on macrophages.

The identity of at least one receptor has been revealed in recent studies using mice deficient for TLR-7 or the

MyD88 signalling pathway (activated by ligand binding of TLR-7). Hemmi and colleagues[17] have shown, using MyD88 or TLR-7 deficient mice, that in the absence of TLR-7 or the signalling pathway, treatment with imiquimod, or its more potent derivative resiquimod (R-848), does not induce the production of inflammatory cytokines, particularly IFN. In consequence, key transcriptional molecules such as NF-κB and Jnk are not activated. The demonstration of this ligand receptor interaction provides a sound mechanistic explanation for the antiviral effects of imiquimod and its homologues and, further, gives important insights into the role of TLR receptors in viral infections. There is little published information on the role of TLR family members in viral infections. In humans, plasmacytoid dendritic cell precursors (pDC2s) can produce IFN-α and IFN-β in response to virus and have been identified as natural IFN producing cells. Intriguingly, of the 10 known TLR receptors, only TLR-7 and TLR-9 are expressed on pDC2s.[18] Interestingly, CpG DNA can stimulate pDC2 to generate IFN (albeit less effectively than imiquimod),[19] and DNA vaccines have been shown to be highly effective in natural papillomaviral infections in animals either prophylactically or therapeutically.[20]

Imiquimod and human papillomavirus (HPV)-associated disease

Spontaneous regression of genital warts is accompanied, histologically, by a dense mononuclear cell infiltrate into the lesion. Immunohistochemical studies show that this infiltrate is dominated by CD4+ T cells although many CD8+ T cells are present.[21] The infiltrating T cells express activation markers such as CD25 and the keratinocytes of the regressing lesions express HLA-DR and intercellular adhesion mollecule (ICAM)-1. Overall, the evidence is that wart regression is associated with a Th1-type response, although the actual cellular effectors in this response still have to be identified unequivocally. Imiquimod, which has been shown to be significantly more effective than placebo in clearing genital warts, activates macrophages and directly induces Th1-type cytokines including IL-12.[9] The ability to generate and maintain effective HPV-specific cell mediated immunity (CMI) may explain in part the efficacy and the low recurrence rates reported in several large clinical trials.[22–24]

This supposition is supported by the results of a double-blind placebo-controlled human mechanism of action study.[25,26] In this, the effects of topically applied imiquimod (Aldara™) or vehicle on wart cytokine mRNA, keratinocyte phenotype and HPV load were examined. Biopsies were taken at 0, 6 and 16 weeks, usually end of treatment (EOT), and HPV DNA copy number was determined by PCR, semiquantitative RT-PCR used to determine relative mRNA expression of cytokine and keratinocyte markers and immunohistochemistry used to define wart immunocytes. Imiquimod treatment resulted in an increase in mRNA levels for all cytokines analysed except IL-4 and IL-5. Statistically significant increases, however, were observed only for IFN-α and the IFN-inducible gene product 2′5′AS at 6 weeks and for IFN-γ both at 6 weeks and at EOT. Both CD4 and CD8 mRNA levels were increased during treatment, but only CD4 levels were significantly raised in the treated group. Treatment had striking effects on viral load with a decline in HPV copy number at 6 weeks and a significant reduction at EOT. This was mirrored by a reduction in both HPV E7 and L1 mRNA expression and an accompanying increase in the keratinocyte differentiation markers K10 and filaggrin.

Table 2 Proposed mechanisms of imiquimod in HPV infections.

No direct action on virus
Activates DCs, monocytes and macrophages via TLR-7
Induces pro-inflammatory cytokines
Activates cell-mediated immunity

The efficacies of 1 and 5% imiquimod cream in different dosing regimens have been examined in several large double-blind placebo-controlled randomized trials. In the pivotal phase III trial,[24] wart clearance in the group treated with 5% cream 3 times weekly was statistically greater than that for placebo (50 vs. 11%, $P < 0.0001$). In general, female patients had a better response than men and showed a more rapid clearance, an effect ascribed to the lower degree of keratinization at wart sites in women.

It is clear that imiquimod induces a strong Th1 response in individuals with condylomata, but 20–40% patients fail to respond. There are many possible explanations for this. Patients with persistent disease may have established tolerance to viral infection, there may be signalling defects in the T cells, or antigen load in the lesions may simply be too low for effective priming. Inadequate DC activation may be a factor, and there is evidence that in imiquimod-resistant genital warts dermal DCs and macrophages are significantly reduced (Table 2).[27]

Imiquimod in the treatment of common warts and molluscum contagiosum

At present, Aldara™ is licensed for use only on condylomata but has been used off-label for the treat-

ment of common warts and other viral skin diseases. Good results have been reported in an open-label phase II trial of 50 patients with recalcitrant warts, 18 of whom were immunosuppressed either as a consequence of HIV infection or post transplantation.[28] Fifteen patients (30%) showed complete clearance after a mean treatment period of about 9.5 weeks. Response to treatment, interestingly, was not influenced by HIV status. Fifteen patients in the trial had molluscum contagiosum and eight of these (50%) cleared completely. Adverse effects were few and recurrences over a 12-month follow-up period low (7% for warts and 8% for molluscum). Several case reports document responses to topical imiquimod in patients with recalcitrant common warts[29–33] and/or molluscum.[34–37] It is of interest that a significant number of immunosupressed individuals with molluscum have responded to imiquimod treatment.

Imiquimod anti-tumour effects: imiquimod and cutaneous neoplasia

In mouse models, long-term protection with imiquimod treatment against tumour challenge has been shown in several studies, and demonstrated to be a consequence of IFN induction.[20] Basal cell carcinoma (BCC) is the most common cutaneous malignancy and, furthermore, is a tumour known to respond to IFN treatment. Therapeutic responses of BCC to imiquimod have been shown in several trials. In a double-blind randomized pilot study, the safety and efficacy of imiquimod 5% cream vs. vehicle was examined.[3] Twenty-four patients received drug and 11 vehicle for up to 16 weeks; excisional biopsies were taken 6 weeks after end of treatment for histological examination. One hundred per cent of patients (15) dosed twice daily, once daily and three times weekly, 60% (three of five) of patients dosed twice weekly and 50% (two of four) of patients dosed once weekly cleared completely on the basis of histological examination compared to 9% (one of 11) of vehicle-treated patients. In a larger multicentre, randomized, open-label, dose–response trial these results were confirmed,[38] suggesting that imiquimod 5% cream has potential as a patient-administered option for BCC.

Intra-epithelial neoplasia of skin and mucosal surfaces has been associated, at both cutaneous and mucosal sites to a variable degree, with HPV infection. At all sites these lesions can present therapeutic problems; the lesions are often multifocal, surgical excision can be difficult or even mutilating, and recurrences are frequent irrespective of the treatment modality. Promising results have been reported with imiquimod 5% cream in the treatment of these lesions. In a phase II open-label study, 16 patients with Bowen's disease self-treated once daily with 5% imiquimod cream.[39] At the 6-week post-treatment biopsy 14 had no residual tumour present. Several case reports document successful treatment of Bowenoid papulosis on ano-genital skin in both immunocompetent[40–43] and immunosuppressed patients.[44,45] Similarly, case reports outline successful outcomes of treatment for several premalignant conditions – actinic keratosis,[46] lentigo maligna,[47] stucco keratosis,[48] and porokeratosis of Mibelli.[49] The mechanisms by which imiquimod exerts an antitumour effect are uncertain but are probably a consequence of cytokine-induced apoptosis. It is clear that double-blind, randomized, placebo-controlled trials are required to define the clinical utility of the imidazoquinolones in the treatment of intraepithelial neoplasia on both cutaneous and mucosal sites. This will require multicentre trials, but the results could be of significant value in the treatment of diseases that present significant clinical problems.

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