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Immune responses induced by intranasal imiquimod and implications for therapeutics in rhinovirus infections

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Abstract

Notwithstanding the progress recently made in immunology and virology, there is yet no effective, specific treatment for the common cold. Symptomatic treatment is minimally effective. An anecdotal report of rapid clearing of the common cold of recent onset after intranasal application of imiquimod in several subjects by one of the authors, made us test the hypothesis that this treatment works through the secretion of interferon by the nasal mucosa. We decided to do an animal study in primates (Indian Macaca Mulata): 5 treatment and 3 control animals were used. Imiquimod or placebo was massaged into the nares of the animals and periodic samples of post-nasal fluid were taken and measurements for Interferon α (IFNα) and Tumor Necrosis Factor α (TNFα) were made by ELISA methods, and kinetic studies. IFNα mRNA was also isolated and analyzed by quantitative competitive RT-PCR. The internal standard was constructed to be complementary to and compete with oligonucleotide primers and for amplification of target sequences. One intranasal application of imiquimod rapidly (1–4 h) induced high levels of mRNA for IFNα, and minimal levels in the control animals. Rapid induction of INFα, and proportional increase of TNFα sustained for 4 and 6 h respectively were noted. No adverse reactions to treatment were found in macaques during this short period of intranasal imiquimod usage (except in one macaque with a short period of lacrimation). No animal had cytotoxic effects when examined at 6 h, 12 h, 24 h or 48 h, except one animal, which had an episode of lacrimation for 6hr post treatment. Thus both safety and efficacy of short treatment with imiquimod is proven in this animal model. Proof of principle for intranasal treatment of the common cold with imiquimod is shown. We think that this work will encourage a number of double blind clinical trials to confirm the effectiveness of the intranasal treatment of the common cold with imiquimod.

Keywords: common cold • Imiquimod • interferon- α • intranasal antiviral treatment

Introduction

The common cold is a very "common" affliction. Millions of people are affected each year. The economic toll is enormous, both in direct cost of medication as well as indirect costs such as lost work and school days. Human rhinoviruses are the most frequent cause of the common cold [1], and the viral rhinitis usually has to run its course for 1–2 weeks. Symptomatic treatment is all that is now available. Imidazoquinolone compounds like imiquimod have been shown to be immunomodulators and are used as topical therapy for some viral infections [2–5]. We hypothesize that intranasal topical immunotherapy with an immunomodulator inducing IFN-α under very controlled conditions may limit the acquisition of the virus, and thus could be a practical and "patient-friendly" treatment for these viral infections. The imidazoquinolone compounds, of which imiquimod (formulated, as Aldara ™), is the best characterized to date, are immuno-modulators molecules which act by activating macrophages and other cells by binding to cell interface receptors like Toll receptor 7, and inducing secretion of inflammatory cytokines, pre-

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dominantly IFNα, tumor necrosis factor TNFα, and interleukins [2]. Toll receptors are a family of pattern recognition receptors recognizing either conserved motifs in pathogens or molecules derived from the host. Their identification provides a new way to activate immune responses to pathogen proteins, by targeting dendritic cells *in situ*. In this approach, the toll receptors ligand is administered intra-dermally or intra-muscularly, encounters dendritic cells and other responsive sentinel cells of the immune system, and activates them by inducing the release of pro-inflammatory cytokines. To date, 11 mammalian toll-like receptors (TLR) have been identified. They are expressed primarily on macrophages and dendritic cells, but have also been found on T and B cell subsets and on keratinocytes. The toll-like receptors are characterized by the presence of an extracellular domain containing leucine-rich repeats, and a cytoplasmic Toll/IL-1 receptor domain. They share a common signal pathway involving the adaptor molecule myeloid differentiation factor 88, which leads to the activation of MAP kinases and NF-κB. Toll-like receptors ligands control the activation of antigen presenting cells, in particular dendritic cells, by triggering their maturation program, including up-regulation of the expression of HLA and co-stimulatory molecules and secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IFN-α [2].

Until recently, the natural ligands of TLR7 and TLR8 were unknown. However, mRNA molecules from vesicular stomatitis virus, rhinovirus and influenza virus were shown during 2004 to be agonists for TLR7. At much the same time, imidazoquinoline compounds like Imiquimod were shown to be TLR7 and TLR8 ligands. Imiquimod (1-(2-methylpropyl)-1H-imidazo [4,5-*c*]quinolin-4-amine, also known as R-837 and S-26308) has been known for many years to have potent anti-viral and anti-tumor effects.) Imiquimod (1-(2-methylpropyl)-1H-imidazo[4,5-*c*]quinolin-4-amine, has been known for many years to have potent anti-viral and anti-tumor effects. Imiquimod was originally identified in a drug screen for anti-herpes virus activity in the1980's, and its first recorded effects as an antiviral agent active against herpes simplex virus in guinea pigs date back to 1989.

Imiquimod, formulated as a 5% vol/vol cream (AldaraTM from 3M Pharmaceuticals Inc), is now clinically approved for the treatment of external genital and perianal warts induced by human papillomavirus. Imiquimod binds to toll-like receptor 7 and therefore activates two major subsets of human blood dendritic cells, *i.e.* the plasmacytoid DC and the $CD11c^+$ myeloid DC. The antiviral activity of imiquimod in the treatment of genital warts is considered to occur through induction of the innate immune response and then the activation of adaptive immunity. Indeed, regression of imiquimod responsive warts is accompanied by increased lymphocyte infiltration into lesions, keratinocyte activation (increased DR, ICAM-1 and IL-12 p40 expression), and evidence of dendritic cell activation.

Aldara cream, to date, has been used as a topical therapy for genital warts [3], molluscum contagiosum [4], basal cell carcinoma [5–6], Bowen's disease [7] and premalignant conditions [8–9]. We chose imiquimod because; 1) it is a registered drug, and therefore, directly available; 2) Imiquimod inhibits replication of HPV *in vitro*, and; 3) in animal studies IFN-α mRNA and TNF-α were detected following intra-vaginal topical application [10–13]. We limited our investigation to examining nasal secretions following nasal application (both nares) of imiquimod, hypothesizing that this will be the entry site where an immune response will be first observed [14].

Methods

Macaque studies

Approval for all animal experiments was obtained from the Tulane University Animal Care and Use Committee. Two groups of Indian Macaca Mulata verified to be free of simian immunodeficiency virus (SIV) and simian retrovirus type D (SRV) infections were used. The control group (n = 3) and the treated group (n = 5) were sedated with glycopyrolate 0.01 mg/kg + acetaminophen 0.2 mg/kg and anesthetized with zolazepam (Telazol) 10 mg/kg intramuscular. Animals were removed from their respective cages and placed on absorbent pads. Each animal was weighed, the hair under the nares was shaved and sample "0" from the post-nasal fluid was taken by inserting thin sticks with cotton swabs deep into the nares. The swabs were then re-inserted in the sterile collection tubes containing transport media Hank's balanced salt solution with 10% glycerol and 200 U/mg each of penicillin and streptomycin, 250 mg/ml gentamycin and 50 U/mg nystatin) [15]. In the treatment group, 1/2 packet of Aldara cream was massaged gently under each nare. A packet of Aldara contains 0.25 g imiquimod and a base cream consisting of isostearic acid, cetyl alcohol, white petroleum, polyphorbate 60, glycerin, benzyl alcohol and propylparaben. In the control group, only the cream was massaged. Samples were taken at different times and stored at 4°C for 1 day and at -20ºC for one week. Vital signs were taken after each sample point by a veterinarian. Physical examination after each sample and at 6 h, 12 h, 24 h, and 48 h post anesthesia consisted of examining each macaque for fever, erythemia, edema, erosion, flaking, and lacrimation. The

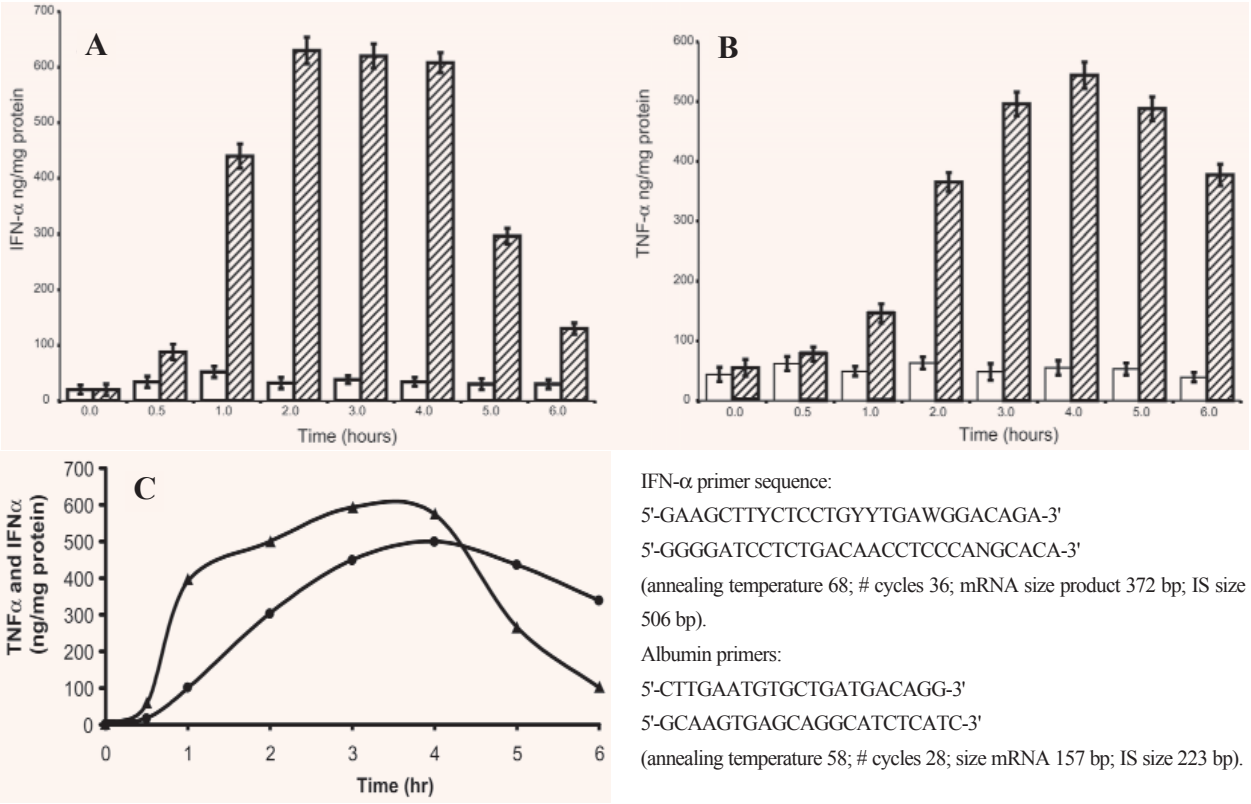

Fig. 1 Time course of IFN-α (A) and TNF-β (B) induction following nasal application of Imiquimod (n=5) ▨ or only base cream in controls (n=3) (C) Kinetics of cytokine induction by nasal application of Imiquimod in macaques (n=4). σ IFN- α; λ TNF-α

animals were followed for 1 week for change in weight, eating habits, stool consistency, and fever. Blood was drawn for complete blood counts and chemistry panels, at 24 h and one week post anesthesia. In one animal a 2.5 mm punch biopsy on the nasal mucosa was performed at 24 h post imiquimod cream application followed by hemostasis with Gelfoam and packing.

RNA isolation

RNA was isolated with a high pure RNA isolation kit (Roche, Molecular Biochemicals) according to the instructions from the manufacturer.

Quantification by competitive RT-PCR

Two μg total cellular RNA was reverse transcribed. Quantification of cDNAs corresponding to transcripts of interest were performed by using internal cDNA standards (IS). In brief, IS were constructed to be complementary to and compete with oligonucleotide primers and for amplification of target sequences. Target cDNAs were amplified in the presence of 10- and two-fold serial dilutions of the IS. The amount of target transcripts was then calculated on the basis of the known molecular quantity of the IS, and related to the amount of a reference mRNA (albumin, β-actin, or glyceraldehyde phosphate dehydrogenase (GAPDH)), which had been quantified in parallel (16,17).

IFN-α primer sequence:
5'-GAAGCTTYCTCCTGYYTGAWGGACAGA-3'
5'-GGGGATCCTCTGACAACCTCCCANGCACA-3'
(annealing temperature 68; # cycles 36; mRNA size product 372 bp; IS size 506 bp).
Albumin primers:
5'-CTTGAATGTGCTGATGACAGG-3'
5'-GCAAGTGAGCAGGCATCTCATC-3'
(annealing temperature 58; # cycles 28; size mRNA 157 bp; IS size 223 bp).

IFN a and TNF a enzyme-linked immunoabsorbent assays (ELISA)

Levels of IFN α and TNF α were measured using commercially available multispecies kit (PBL Biomedical Laboratories) (product #41105-1). The range of detection was 10-500 pg/ml (high sensitivity protocol).

Statistical analysis

Data were compared using student t-test or one-way ANOVA and Dunnett's comparison test. Differences were considered significant at $p < 0.05$.

Results

ELISA measurements of IFNα and TNFα (Fig. 1) showed a very significant increase ($p < 0.0001$) over time of both cytokines (IFNα to 630 ng/mg protein (Fig. 1A) TNFα to 540ng/mg protein) (Fig. 1B) as compared with a minimal and steady concentration of IFN and TNF in the control group (Figs. 1A–1B).

Kinetic studies of IFNα and TNFα levels (Fig. 1 C) showed a very rapid induction of IFNα even at 1 h post treatment increasing to 5 times basal level at 3 h, remaining near maximal level at 4 h, but decreasing rapidly to near basal level at 6 h post treatment. TNFα also increased proportionally, but slightly less than IFNα peaking at 4 h, but remaining high even at 6 h post treatment. The significance of these data is two-

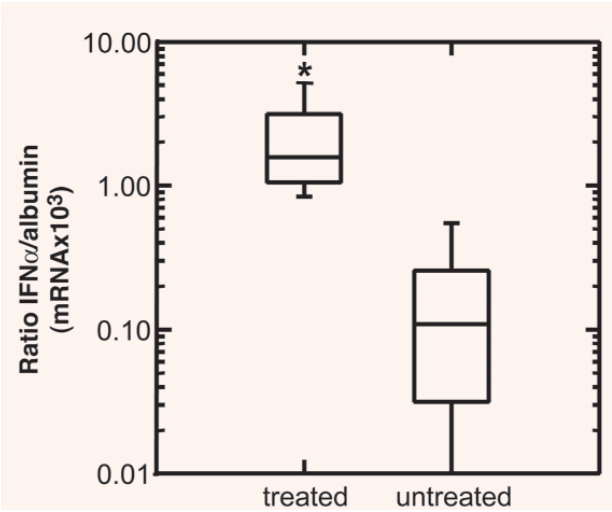

Fig. 2 Intranasal expression of IFN-α mRNA in macaques untreated or treated by nasal application of Imiquimod. Boxes show all results in a group (n=4). Medians indicated by horizontal bars; S.D. by vertical bars. *Level of significance is given for mRNAs in relation to albumin mRNAs by using quantitative RT-PCR assays (p=0.04)

fold. First, intranasal administration of imiquimod cream (5%) produced enough IFNα in a short period of time, so the cream can be applied daily in order to have 5–6 h of good efficacy. However, the cream has known cytotoxic effects (mediated through the cytokine production), especially if applied for weeks, so extended application would not be recommended [2]. In our experiment TNFα remained high for at least 6h. In the treated group of macaques, the cream was washed away with warm water after the experiments. No animal had cytotoxic effects when examined at 6 h, 12 h, 24 h or 48 h, except one animal, which had an episode of lacrimation for 6 h post treatment. No long-term local toxic effects were seen by histopathological examination of the nasal punch biopsy nor did any safety problems appear at least as seen by physical examinations or routine laboratory evaluations. Thus, the second point is that the cream only needs to be applied to induce maximum concentration of IFNα but it may be removed when TNF-α continues to be elevated (after 6 h).

The final set of studies evaluated mRNA IFN induction by imiquimod in post-nasal swabs. mRNA was isolated and analyzed by quantitative competitive RT-PCR. We used an internal standard constructed to be complementary to and to compete with oligonucleotide primers and for amplification of target sequences. We used samples 3–5 h post-nasal application. IFNα protein expression was induced by imiquimod at levels 2–5 times the control samples (Fig.2). IFNα mRNA was also variably induced in control animal samples (anesthesia only) at ratios between 0.01-0.55 with a median of 0.192. The treated animals expressed IFNα mRNA at a level of 0.84-5.20 mRNA x 10^3 with a mean of 2.3 (Fig.2). The difference was significant ($p = 0.04$) irrespective of whether IFNα mRNA levels were related to albumin, β–actin or GAPDH as reference transcripts. Whether high mRNA levels of IFNα and thus, immunostimulatory effects of imiquimod are seen after 6 h of nasal application remains to be determined.

Discussion

Taken together, these results show that nasal application of Imiquimod will induce rapid (1–4 h) and appreciable amounts of IFNα locally. Also, IFNα therapy with Imiquimod against acute viral infection with rhinovirus may be most effective when administered before the peak of virus replication [17–19]. The significance of these data is twofold: First, intranasal administration of Imiquimod cream stimulated production of considerable amounts of interferon in a short time. Second, the effect was prolonged. Thus, if the cream is applied every 24 h, one can have a sustained response. As the cream has known cytotoxic effects mediated by cytokine production (in vaginal and rectal prolonged application) [2] extended application (more than 3 days) is not recommended. However, the time interval for effective treatment with Imiquimod in humans may be longer that in experimental animals. The nasal route of application has demonstrated great potential for inducing both humoral and cell-mediated immunity in mucosa as well as in the systemic compartment.

It is known that Imiquimod also induces true cell mediated immunity by helping the dendritic cells mature into antigen presenting cells. Furthermore, it promotes secretion of other pro-inflammatory cytokines, in addition to TNFα such as interleukin 12. Thus, a Th1 immune response is favored. [2] In addition, through induction of interleukins 6 and 10, natural killer cells are activated as well as interferon production, while by stimulation of production of interleukin 8, B-cell activation takes place [20]. Therefore the effectiveness of imiquimod cream may be due to its ability to directly activate dendritic cells, B cells, and macrophages to induce secretion of Th1 cytokines and chemokines. Stimulation of INFα and TNFα produc-

tion at mucosal surfaces as shown from our data in the macaques may serve to prevent binding or transfer of virus to susceptible co-receptor bearing cells.

Intranasal application using imiquimod could be effective both by blocking the virus at the site of entry and in inducing antibodies in the respiratory tract. Besides if virus infection can be blocked at the site of entry there may be less risk for the immune enhancer drug to develop antibody enhancement phenomena. Even if the virus broke through this front-line, serum-neutralizing antibodies could further interact and neutralize the virus.

Treatment has to start within 24 h of the onset and preferably within 12 h of symptoms when the viral load is smaller than it is late in the infection. It may be anticipated that in some cases treatment may fail, if there is a low density of dendritic cells at the site of application of Imiquimod. [21].

We believe that these preliminary results in the primate model warrant further clinical studies with nasal application of Imiquimod in rhinovirus infections as a potentially effective treatment or even preventive treatment.The safety profile as seen by an application of only 6 h, the fact that nasal mucosa biopsy was unremarkable after 24 h, shows that a shorter application may be both safe and effective. It is apparent that rhinoviruses enter cells and fuse within endosomal compartment. This step in the viral life cycle is amenable to inhibition for antiviral therapies. Thus, prophylactic or early post exposure treatment may also be investigated to help reduce the impact of viral rhinitis on health-care workers, and others possibly exposed, and therefore, limit the spread of the virus in the population in general, and school age populations in particular.

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