Cis-urocanic acid, a sunlight-induced immunosuppressive factor, activates immune suppression via the 5-HT_{2A} receptor

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Exposure to UV radiation induces skin cancer and suppresses the immune response. To induce immune suppression, the electromagnetic energy of UV radiation must be absorbed by an epidermal photoreceptor and converted into a biologically recognizable signal. Two photoreceptors have been recognized: DNA and *trans*-urocanic acid (UCA). *Trans*-UCA is normally found in the outermost layer of skin and isomerizes to the cis isomer upon exposure to UV radiation. Although UCA was identified as a UV photoreceptor years ago, and many have documented its ability to induce immune suppression, its exact mode of action remains elusive. Particularly vexing has been the identity of the molecular pathway by which *cis*-UCA mediates immune suppression. Here we provide evidence that *cis*-UCA binds to the serotonin [5-hydroxytryptamine (5-HT)] receptor with relatively high affinity (K_d = 4.6 nM). Anti-*cis*-UCA antibody precipitates radiolabeled 5-HT, and the binding is inhibited by excess 5-HT and/or excess *cis*-UCA. Similarly, anti-5-HT antibody precipitates radiolabeled *cis*-UCA, and the binding is inhibited by excess 5-HT or excess *cis*-UCA. Calcium mobilization was activated when a mouse fibroblast line, stably transfected with the human 5-HT_{2A} receptor, was treated with *cis*-UCA. *Cis*-UCA-induced calcium mobilization was blocked with a selective 5-HT_{2A} receptor antagonist. UV- and *cis*-UCA-induced immune suppression was blocked by antiserotonin antibodies or by treating the mice with 5-HT_{2A} receptor antagonists. Our findings identify *cis*-UCA as a serotonin receptor ligand and indicate that the immunosuppressive effects of *cis*-UCA and UV radiation are mediated by activation of the 5-HT_{2A} receptor.

immune regulation | inflammation | serotonin | UV radiation

The UV radiation found in sunlight is the primary cause of nonmelanoma skin cancer and is implicated in the induction of malignant melanoma (1). Skin cancer is the most prevalent form of human cancer. The American Cancer Society estimates that over one-half of all cancers diagnosed in the United States are skin cancer. Approximately 1 million cases of skin cancer were diagnosed last year, and ≈10,000 deaths were attributed to skin cancer (www.cancer.org/statistics). In addition to its carcinogenic potential, UV exposure is also immune suppressive. Data from studies with experimental animals and with biopsy-proven skin cancer patients indicate that the immune suppression induced by UV radiation is a major risk factor for skin cancer induction (2, 3). Moreover, UV exposure suppresses the immune response to infectious agents. After a single exposure to doses of UV radiation equivalent to those received during normal human occupational or recreational activities, the immune response of experimental animals (4, 5) or human volunteers (6) to microbial antigens is suppressed. The immune suppression caused by sunlight exposure also plays a significant role in herpes virus recrudescence (7). Because of the association between UV-induced immune suppression and carcinogenesis, and in light of the fact that exposure to UV radiation occurs daily and may be increasing due to the effects of atmospheric pollution on the ozone layer, it is critically important to understand the mechanisms underlying UV-induced immune suppression.

To induce immune suppression, the electromagnetic energy of UV radiation must first be absorbed by an epidermal photoreceptor and then converted into a biologically recognizable signal. Two such epidermal photoreceptors have been identified: DNA (8) and urocanic acid [3-(1H-imidazol-4-yl)-2-propenoic acid; UCA] (9). UCA is located superficially in the stratum corneum. Metabolism of epidermal UCA does not occur *in situ* due to the absence of epidermal urocanase, resulting in the accumulation of UCA in the epidermis. Upon UV exposure, naturally occurring *trans*-UCA converts to the cis isomer, in a dose-dependent manner, until the photostationary state is reached (10). Although UCA was first recognized as a UV-photoreceptor for immune suppression 20 years ago (9), and many have documented its ability to influence immune suppression (11) and carcinogenesis (12), its exact mode of action remains elusive. Particularly vexing has been the identity of the molecular pathway and the cellular receptor by which *cis*-UCA mediates immune suppression.

Once the physical energy of UV radiation is converted into a biologically recognizable signal, that signal must be transmitted to the immune system in order to induce immune suppression. Considerable evidence supports a role for UV-induced biological response modifiers and immune modulatory cytokines in activating immune suppression after UV exposure (13). Although the interplay between these various UV-induced immune modulatory factors is complex and not completely understood, it appears that a cytokine cascade is activated that ultimately induces immune suppression (14). One of the earliest biochemical events in the cascade of events leading to immune suppression is the secretion of platelet-activating factor (PAF) by UV-irradiated keratinocytes. PAF is secreted by epidermal cells almost immediately following UV radiation (15), and injecting PAF in lieu of UV exposure activates immune suppression (16). Similarly, blocking PAF receptor binding with a variety of PAF receptor antagonists blocked cytokine gene transcription and UV-induced immune suppression (16).

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Abbreviations: DTH, delayed-type hypersensitivity; 5-HT, 5-hydroxytryptamine (serotonin); PAF, platelet activating factor; PGE_2, prostaglandin E_2; UCA, urocanic acid.

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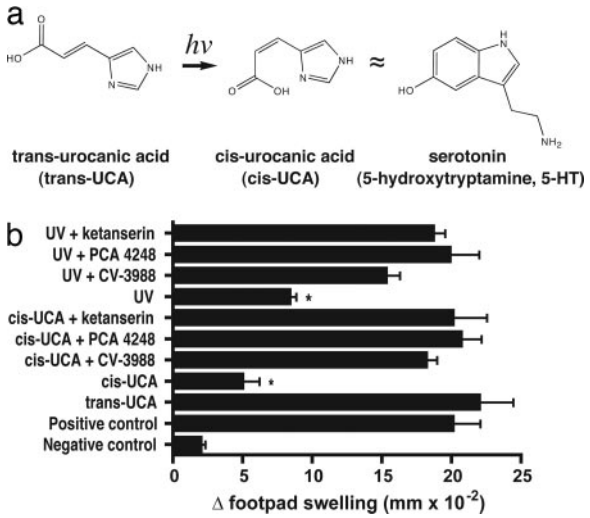


Fig. 1. PAF and serotonin receptor antagonists block *cis*-UCA-induced immune suppression. (*a*) Structural comparison of 5-HT and *cis*-UCA. (*b*) Treating mice with either 500 nmol of PAF or 500 nmol of serotonin receptor antagonists before injecting 5 μmol *cis*-UCA or exposing them to 10 kJ/m^2 of UV radiation blocks the induction of immune suppression. The asterisk denotes a significant difference ($P < 0.01$) from the positive control.

During our investigation into the immunosuppressive properties of PAF, we asked whether PAF receptor binding plays a role in *cis*-UCA-induced immune suppression. We treated mice with *cis*-UCA in the presence or absence of PAF receptor antagonists and measured the immune response (16). In these series of experiments, we included what we thought was an appropriate control, the selective serotonin (5-HT) receptor antagonist, ketanserin (17). Much to our surprise, ketanserin blocked *cis*-UCA-induced immune suppression. This observation suggested that *cis*-UCA and serotonin share the same receptor.

Here we provide data indicating that *cis*-UCA binds to the serotonin receptor. In addition, we report that UV-induced and *cis*-UCA-induced immune suppression were blocked by selective serotonin 5-HT$_{2A}$ receptor antagonists. Our findings identity the cellular receptor by which *cis*-UCA mediates immune suppression and provide insights into the role of serotonin in immune regulation.

Results

Several clues led us to hypothesize that *cis*-UCA might be a serotonin receptor agonist. The work of Ash *et al.* (18) revealed structural clues to the behavior of *cis*-UCA in aqueous solution. Upon isomerization, *cis*-UCA forms a ring-like structure. Analysis by NMR indicates that the ring structure of *cis*-UCA is stabilized by strong intramolecular hydrogen bonding between the inner nitrogen of the imidazole ring and the carboxylic acid moiety. This ring-like structure resembles the structure of serotonin (Fig. 1*a*). Another clue came from our continuing studies on the role of PAF receptor binding in UV-induced immune suppression. Because of the important role *cis*-UCA plays in UV-induced immune suppression, we wished to determine whether blocking of PAF receptor binding would interfere with *cis*-UCA-induced immune suppression. Mice were injected with 500 nmol of the selective PAF antagonists PCA-4248 or CV-3988, as described previously (16). Another group of mice were injected with an equimolar amount of ketanserin, a 5-HT$_{2A}$ receptor antagonist. Thirty minutes later, the mice were injected with an immunosuppressive (5 μmol) dose of *cis*-UCA (19). Five days later, the mice in both groups were immunized with *Candida albicans* and the effect that *cis*-UCA treatment had on the induction of a delayed-type hypersensitivity (DTH) reaction was measured (Fig. 1*b*). As expected, PAF receptor antagonists blocked *cis*-UCA and UV-induced immune suppression. Ketanserin, a selective serotonin receptor antagonist that we used as a negative control in these experiments, unexpectedly also blocked *cis*-UCA and UV-induced immune suppression. Previ-

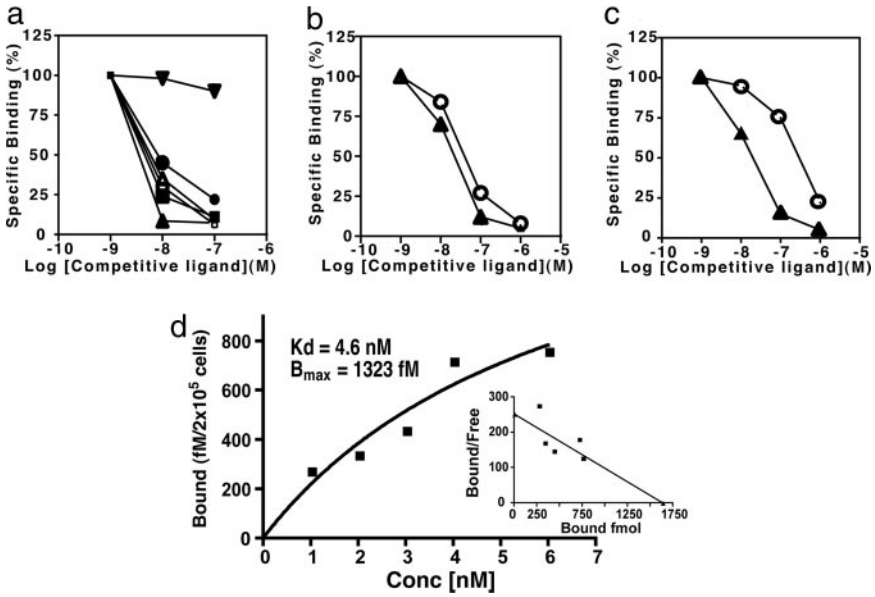


Fig. 2. 5-HT and *cis*-UCA share binding sites. (*a*) Membrane preparations isolated from insect cells expressing the human serotonin receptor were incubated with radiolabeled *cis*-UCA (0.1 μM) or radiolabeled 5-HT (0.1 μM) in the presence of different amounts of nonradioactive ligand. Filled inverted triangles, [^{14}C]5-HT + *trans*-UCA; filled circles, [^{14}C]5-HT + *cis*-UCA; filled squares, [^{14}C]5-HT + 5-HT; open triangles, [^{14}C]5-HT + PCA-4248; M, *cis*-[^{14}C]UCA + PCA-4248; filled triangles, *cis*-[^{14}C]UCA + 5-HT. (*b*) *cis*-[^{14}C]UCA was immunoprecipitated with *cis*-UCA mAb in the presence of increasing amounts of nonradioactive *cis*-UCA (open circles) or nonradioactive 5-HT (filled triangles). (*c*) *cis*-[^{14}C]UCA was immunoprecipitated with anti-5-HT antibody in the presence of increasing amounts of nonradioactive *cis*-UCA (open circles) or nonradioactive 5-HT (filled triangles). (*d*) Saturation binding of *cis*-[^{3}H]UCA to L-NCG-5HT$_{2A}$ cells. Data points represent specific binding; K_d and B_{max} were determined from the best-fit nonlinear regression curve of the saturation isotherm. The saturation binding curve was converted to a Scatchard plot.

ous experiments indicated that injecting the receptor antagonists by themselves did not affect DTH (20). This observation suggested that *cis*-UCA and serotonin share the same receptor, prompting a direct test of the hypothesis.

Competitive binding experiments were used to determine the ability of radiolabeled *cis*-UCA to bind to the serotonin receptor. Membrane preparations, isolated from Sf9 insect cells transfected with the human serotonin receptor, were incubated with 0.1 μM *cis*-[^{14}C]UCA in the presence or absence of 10^{-6} to 10^{-9} M nonradioactive *trans*-UCA, *cis*-UCA, or 5-HT (Fig. 2*a*). Excess *cis*-UCA and serotonin, but not *trans*-UCA, displaced the binding of *cis*-[^{14}C]UCA to the 5-HT-positive membranes. Treating the membrane preparations with PCA-4248, a selective serotonin receptor antagonist (21), also blocked the binding of radiolabeled *cis*-[^{14}C]UCA to the 5-HT positive membranes. Similarly, when 0.1 μM [^{14}C]5-HT was incubated with the membrane preparations in the presence of nonradioactive *cis*-UCA or 5-HT, the binding of radiolabeled 5-HT to the 5-HT receptor was blocked. Here also, PCA-4248 blocked the binding of [^{14}C]5-HT to its receptor (Fig. 2*a*). These observations indicate that serotonin and *cis*-UCA bind to the same receptor.

Immunoprecipitation studies confirmed the structural similarity of *cis*-UCA and serotonin. *cis*-[^{14}C]UCA (0.1 μM) was incubated with 5 μg/ml anti-*cis*-UCA antibody in the presence or absence of 10^{-6} to 10^{-9} M nonradioactive *cis*-UCA or 5-HT. Precipitation of the radiolabeled ligand was blocked by excess *cis*-UCA and 5-HT (Fig. 2*b*). Similarly, [^{14}C]5-HT was incubated with antiserotonin antibody, and excess amounts of unlabeled *cis*-UCA or serotonin blocked the precipitation of the radiolabeled ligand (Fig. 2*c*). These observations indicate that the ring-like structure formed by trans-to-cis isomerization of UCA (Fig. 1*a*) is immunologically similar to the epitope recognized by the antiserotonin antibody. Saturation binding studies (Fig. 2*d*) indicated that *cis*-UCA binds to the human 5-HT$_{2A}$ receptor with relatively high affinity (K_d = 4.6 nM). This result compares favorably with the binding of serotonin to the 5-HT$_{2A}$ receptor (22).

To determine whether *cis*-UCA could activate cells by means of the serotonin receptor, we measured Ca^{2+} mobilization. L-NCG-5-HT$_{2A}$ cells, or the untransfected control cells, LM(TK$^-$), were loaded with fura-2 acetoxymethyl ester and then treated with 10 μM serotonin, 10 μM *cis*-UCA or 10 μM *trans*-UCA in the presence or absence of a serotonin receptor antagonist. Mobilization of intracellular calcium stores was then monitored. Calcium was released rapidly when the serotonin receptor-positive cells were treated with *cis*-UCA but not after subsequent treatment with *trans*-UCA (Fig. 3*a*). Similarly, L-NCG-5-HT$_{2A}$ cells first treated with *trans*-UCA did not react but did release calcium upon subsequent *cis*-UCA treatment (Fig. 3*b*). Pretreating the L-NCG-5-HT$_{2A}$ cells with 500 nmol of ketanserin completely prevented *cis*-UCA-induced calcium mobilization (Fig. 3*c*). As a positive control, L-NCG-5HT$_{2A}$ cells were treated with 5-HT, which clearly induced calcium mobilization (Fig. 3*d*). No calcium flux was observed when the serotonin receptor-negative cell line LM(TK$^-$) was treated with *cis*-UCA (Fig. 3*e*). These data indicate that *cis*-UCA and serotonin bind to and activate cells by engaging the 5-HT$_{2A}$ receptor.

Next we examined the effects of a serotonin-specific antibody, or antibody specific for *cis*-UCA, on UV-, *cis*-UCA-, or 5-HT-induced immune suppression (Fig. 4*a*). Mice were injected with 5 μg of anti-*cis*-UCA or 5 μg of anti-5-HT antibody, or an equivalent amount of isotype-matched control antibody, 30 min before UV exposure (10 kJ/m^2 UVB radiation), treatment with *cis*-UCA (5 μmol, i.p.), or treatment with serotonin (250 pmol, i.p.). Five days later, the mice were immunized with *C. albicans*, and DTH *in vivo* was used to measure the effect that each of these treatments had on the immune response. As expected, exposing the mice to UV or injecting them with *cis*-UCA suppressed immunity. Similarly, DTH was suppressed when mice were injected with 250 pmol serotonin,

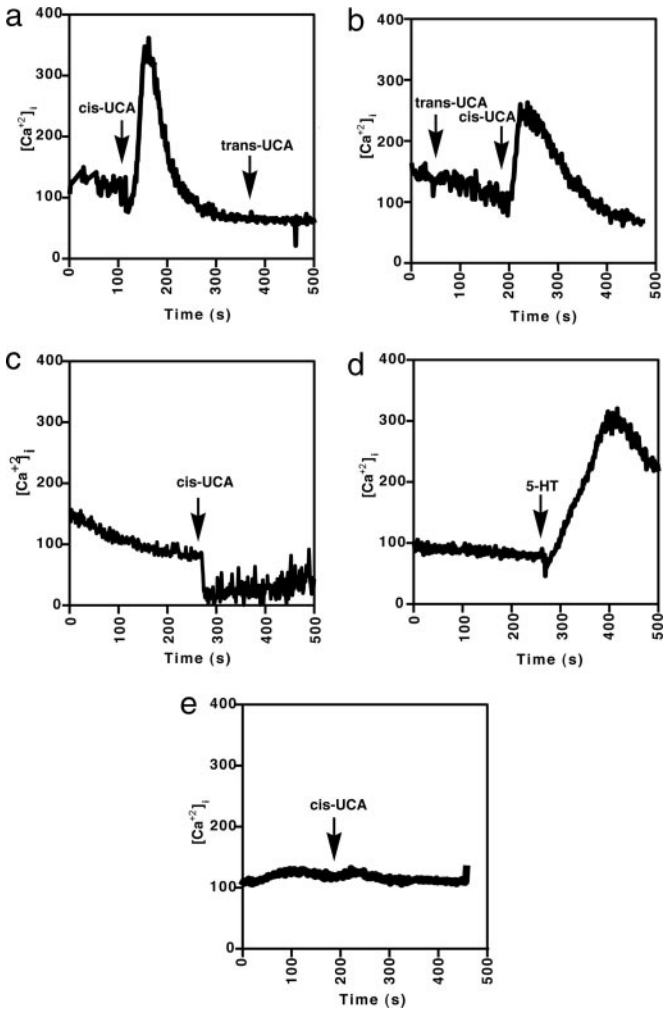
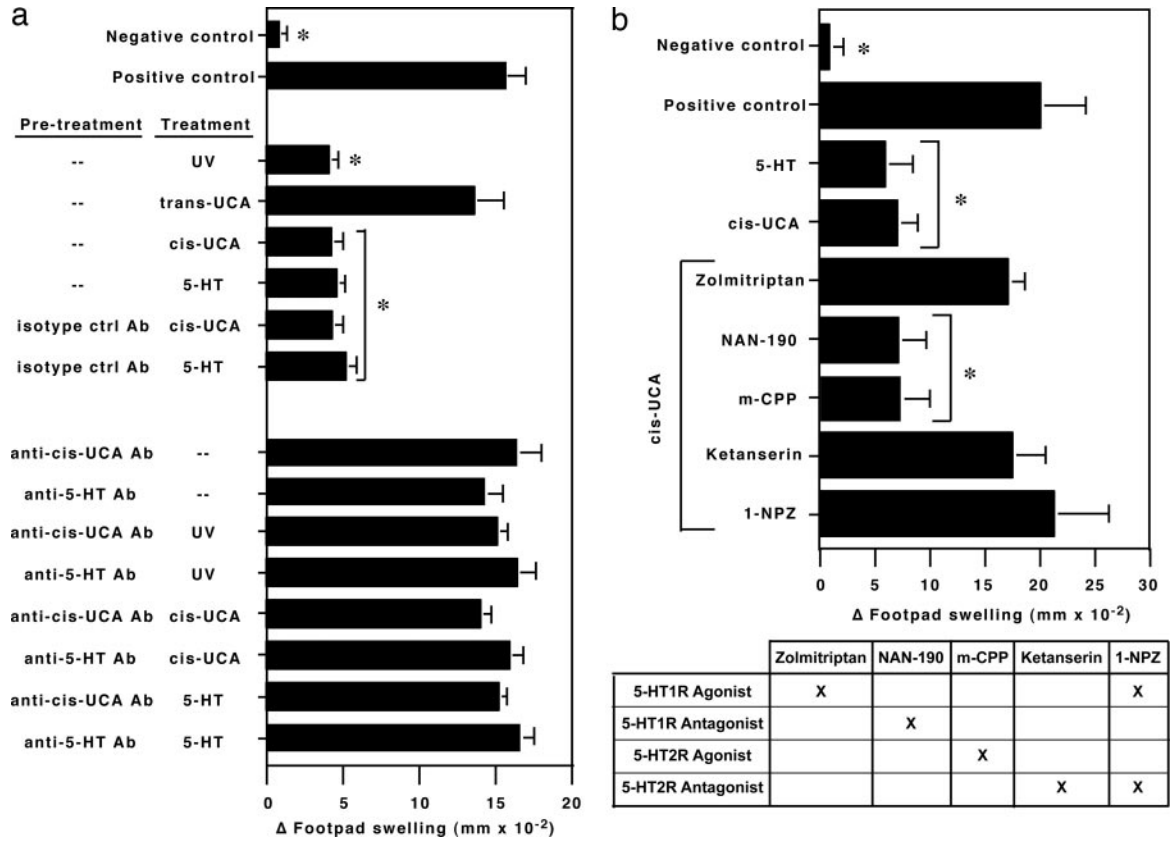


Fig. 3. *cis*-UCA activates intracellular calcium flux via the serotonin receptor. (*a* and *b*) Calcium flux was induced in L-NGC-5HT$_{2A}$ by adding *cis*-UCA but not *trans*-UCA. (*c*) Pretreating the cells with ketanserin, a 5-HT$_{2A}$ receptor antagonist, blocks *cis*-UCA-induced calcium flux. (*d*) Positive control, L-NGC-5HT$_{2A}$ cells treated with 5-HT. (*e*) Negative control, LM(TK$^-$) cells treated with *cis*-UCA.

a physiological dose encountered during inflammation (23) (Fig. 4*a*). Confirming previous findings, we found that UV- or *cis*-UCA-induced immune suppression was blocked in mice treated with monoclonal anti-*cis*-UCA antibody (19). Similarly, the immune suppression induced by injecting 5-HT into mice was blocked when the mice were treated with anti-5-HT or anti-*cis*-UCA. The antibodies by themselves, when injected in mice that were not treated with UV, 5-HT, or *cis*-UCA, had no effect on DTH (Fig. 4*a*).

Because there are 14 known 5-HT receptor subtypes, and because at least 8 are expressed on immune tissues (24), we wanted to determine whether blocking/activating different classes of serotonin receptors had an effect on *cis*-UCA-induced immune suppression (Fig. 4*b*). In this experiment, mice were injected with 500 nmol of the various serotonin receptor agonists or antagonists 30 min before they were injected with an immunosuppressive dose of *cis*-UCA. As expected, ketanserin blocked *cis*-UCA-induced immune suppression. Interestingly, pretreating the *cis*-UCA-injected mice with the antimigraine drug zolmitriptan, a 5-HT$_{1B/D}$ receptor agonist (25), reversed *cis*-UCA-induced immune suppression. Similarly, the dual 5-HT$_1$ agonist/5-HT$_2$ antagonist (1-NPZ) (26) prevented *cis*-UCA-induced immune suppression. On the other hand, pretreating the *cis*-



	Zolmitriptan	NAN-190	m-CPP	Ketanserin	1-NPZ
5-HT1R Agonist	X				X
5-HT1R Antagonist		X			
5-HT2R Agonist			X		
5-HT2R Antagonist				X	X

Fig. 4. Inhibition of *cis*-UCA- and UV-induced immune suppression with selective 5-HT receptor antagonists, anti-*cis*-UCA antibody, or anti-5-HT antibody. (*a*) Treating mice with 5 μmol *cis*-UCA, 10 kJ/m^2 of UV radiation, or 250 pmol 5-HT induces immune suppression. Pretreating the mice with anti-*cis*-UCA mAbs or with anti-5-HT mAb before UV exposure, 5-HT-treatment, or *cis*-UCA treatment blocks the induction of immune suppression. (*b*) Treating mice with the 5-HT$_{2A}$ receptor antagonists (ketanserin and 1-NZP) and with a 5-HT$_1$ receptor agonist, zolmitriptan, blocks *cis*-UCA-induced immune suppression. The 5-HT$_1$ receptor antagonist NAN-190 and the 5-HT$_{2C}$ receptor agonist *m*-CPP were ineffective in blocking *cis*-UCA-induced immune suppression. An asterisk indicates a significant difference ($P < 0.01$) from the positive control.

UCA-injected mice with *m*-CPP, a 5-HT$_{2C}$ receptor agonist (27), or with NAN-190, a 5-HT$_1$ receptor antagonist (28), was ineffective in blocking *cis*-UCA-induced immune suppression. These data support our hypothesis that *in vivo* activation of the 5-HT$_{2A}$ receptor suppresses DTH.

Discussion

The observation that *cis*-UCA, an immune modulator produced in the stratum corneum, induces immunosuppression by binding to the 5-HT$_{2A}$ receptor provides insight into sunlight-induced immune suppression, sunlight-induced carcinogenesis, and the immunomodulatory role of serotonin. Our data indicate that serotonin receptor engagement can suppresses the immune response. This appears to contradict data published by Matsuda *et al.* (29), who showed that the vasoactive effects of serotonin were critically important for the efflux of immune cells into the site of antigen deposition during the challenge phase of DTH. A major difference in the way the experiments were designed may help to explain this discrepancy. In our experiments, serotonin/*cis*-UCA/UV exposure was given 5 days before immunization and 15 days before challenge. We propose that serotonin or *cis*-UCA given well before challenge activates processes known to be critical for UV- and *cis*-UCA-induced immunosuppression, such as mast cell activation (30, 31) and the secretion of antiinflammatory cytokines (14) that normally serve to dampen hypersensitivity reactions. UV exposure maybe acting *in vivo* to accelerate these normal feedback mechanisms, thereby promoting immune suppression. Alternatively, serotonin receptors may be desensitized (32), or serotonin transporters activated (33), when UV or *cis*-UCA is given 15 days before antigenic challenge.

Although our analysis was limited, our findings suggest that the activation/inhibition of different serotonin receptor subtypes may have different effects on the induction of immune suppression. This observation was not too surprising because it is known that activation of different 5-HT subtypes has diverse immunological consequences. For example, 5-HT$_1$ receptor binding stimulates T and B cell proliferation (24). Activation of the 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors activates monocytes to secrete cytokines (34). Our findings are consistent with the concept that differential 5-HT receptor activation may induce different immunological consequences. We found that 5-HT$_{2A}$ receptor antagonism blocked UV- and *cis*-UCA-induced immune suppression and that treating *cis*-UCA-treated mice with a 5-HT$_1$ receptor agonist (zolmitriptan) also blocked immune suppression. Similarly, 1-NZP, which is both a 5-HT$_{2A}$ receptor antagonist and a 5-HT$_1$ receptor agonist, blocked immune suppression. Whether the reversal of immune suppression by 1-NZP is simply due to the inhibition of *cis*-UCA binding to the 5-HT$_{2A}$ receptor or is due to an activation of immune cells via the 5-HT$_1$ receptor remains to be seen.

The downstream immunologic targets of *cis*-UCA are not entirely clear, but the observation that *cis*-UCA binds to the 5-HT$_{2A}$ receptor may shed some light on strategies to address this problem. The 5-HT$_{2A}$ receptors are found on dendritic cells (35). In view of the fact that antigen-presenting cell function is down-regulated after *cis*-UCA treatment and UV exposure (36),

it may be worthwhile to determine whether 5-HT_{2A} receptor antagonists can block depressed antigen-presenting cell function. Alternatively, T and B cells express 5-HT_{2A} receptors (37). It is interesting to note that others have demonstrated that treating activated T cells with *cis*-UCA causes the secretion of IL-10 (38), a cytokine previously found to play a critical role in the immune suppression induced by UV radiation (39). Similarly, a recent report in the literature indicates that total-body UV exposure induces regulatory B cells to secrete IL-10 (40). Whether *cis*-UCA binding to 5-HT_{2A} receptors expressed on T and B cells is responsible for IL-10 production remains to be seen. It is also well known that mast cell activation is critical for UV- and/or *cis*-UCA-induced immune suppression (30, 31). Kahlil *et al.* (41) report that *cis*-UCA does not directly cause degranulation of mast cells, but rather stimulates neuropeptide release from peripheral nerves, which in turn activates mast cells. It is interesting to note that peripheral nerves express 5-HT_{2A} receptors (42). Here again, it is not clear whether *cis*-UCA binding to 5-HT_{2A} receptors on nerve cells is causing the release of neuropeptides and subsequent mast cell activation. Alternatively, *cis*-UCA may be binding to mast cells, stimulating the release of PAF, which then activates the cytokine cascade responsible for UV-induced immune suppression.

Previously, Laihia and colleagues (43, 44) presented data indicating that *cis*-UCA binds to the $GABA_A$ receptor. Although predominately found in the brain, functional $GABA_A$ receptors are also found on T cells, and $GABA_A$ receptor agonists and antagonists have been shown to modulate immune function (45). Although the evidence for *cis*-UCA binding to the GABA receptor is clear, it is not readily apparent that *cis*-UCA is mediating immune suppression through the GABA receptor. Because GABA receptor engagement mediates an inhibitory event in the brain, some have suggested that *cis*-UCA may act as a competitive inhibitor in the periphery to reverse the effects of GABA and allow the production of the prostaglandins and cytokines necessary for UV-induced immune suppression (44). Although injecting GABA into normal mice suppresses DTH to a degree (≈30% reduction) (45), the threshold dose for GABA-mediated inhibition of T cell function (300 μM) is well above the levels of GABA (3 μM) (45) or *cis*-UCA (217 nM) (46) found in the periphery. Moreover, no information is available to indicate whether treating UV-irradiated mice with specific $GABA_A$ receptor antagonists or agonists will modulate UV- or *cis*-UCA-induced immune suppression, unlike the direct evidence we present here indicating a role for 5-HT_{2A} receptor binding in UV- and *cis*-UCA-induced immune suppression.

The role of histamine in UV- and *cis*-UCA-induced immune suppression is well documented (47). Given the structural similarities between histamine and serotonin, a question that arises is whether *cis*-UCA binding to histamine receptors is responsible for immune suppression. Two pieces of data suggest that this is not the case. First, Laihia *et al.* (43) found that *cis*-UCA does not bind to histamine receptors. Second, the binding of ketanserin to the histamine receptor is negligible (17), suggesting that reversal of *cis*-UCA- and/or UV-induced immune suppression by ketanserin cannot be attributed to antagonism of histamine receptor binding.

Recently, Woodward *et al.* (48) suggested that serotonin is not the receptor for *cis*-UCA. Unfortunately, unlike the studies described here, Woodward *et al.* failed to directly measure immune suppression after treatment with *cis*-UCA, but rather used monocyte prostaglandin E_2 (PGE_2) secretion as a surrogate endpoint. They report that activation of monocytes with a 5-$HT_{2A/2C}$ receptor agonist fails to induce PGE_2 secretion. Although it is clear that PGE_2 plays a role in UV-induced immune suppression (14), it is not clear that PGE_2 secretion by monocytes is, by itself, solely responsible for inducing immune suppression. Nor should the failure of 5-HT_{2A} activation to stimulate cytokine secretion by monocytes be too surprising because others have shown that monocyte cytokine secretion is activated by 5-HT_3, 5-HT_4, and 5-HT_7 receptor binding (34) and suppressed by 5-HT_{2A} receptor binding (49). Based on these observations, we stand by our conclusion that the receptor involved in *cis*-UCA-induced immune suppression is 5-HT_{2A}.

Methods

Reagents. UCA was purchased from Acros Organics (Geel, Belgium). Ketanserin, NAN-190, 1-(1-naphthyl) piperazine hydrochloride, 1-(*m*-chlorophenyl)piperazine, serotonin, antiserotonin antibody, histidase, and L-histidine were purchased from Sigma-Aldrich (St. Louis, MO). CV-3988 and PCA-4248 were purchased from Biomol (Plymouth Meeting, PA). Radiolabeled ^{14}C, [^{14}C]serotonin, and [^{3}H]L-histidine were purchased from Amersham Biosciences (Piscataway, NJ). L-NCG-5-HT_2 and the nontransfected parental cell line LM(TK^-) were purchased from American Type Culture Collection (Manassas, VA).

Preparation of Radiolabeled *cis*-UCA. *trans*-[^{3}H]UCA or *trans*-[^{14}C]UCA was prepared by the enzymatic deamination of radiolabeled histamine, as described previously (50). Radiolabeled *trans*-UCA was irradiated with 500 J/m^2 of UVB radiation (FS-40 sunlamp; National Biological, Twinsberg, OH). The solution was spotted on a prerun silica gel 60 TLC plate (Merck, Darmstadt, Germany) and developed with 40% methanol/60% chloroform. After chromatography, the position of the trans and cis isomers was determined by UV-illumination of the TLC plate and comparison of the migration of the radiolabeled material vs. known standards. The radiolabeled *cis*-UCA was eluted from the silica gel powder by several rounds of chloroform/methanol solvent extraction. The concentration of *cis*-UCA was determined spectrophotometrically, as described by Morrison (10).

Radioligand Binding Assays. Membrane preparations isolated from Sf9 cells that express the human 5-HT receptor were purchased from Sigma-Aldrich. The membrane suspension (500 μl), diluted 1:50 in 50 mM Tris·HCL (pH 7.4) containing 10 mM $MgSO_4$, 0.5 mM EDTA, and 0.1% ascorbic acid was incubated with 0.1 μM *cis*-[^{14}C]UCA or [^{14}C]5-HT in the presence of 10^{-6} to 10^{-9} M nonradioactive ligand. After 3 h at 37°, the receptor–ligand complexes were captured on 3% polyethyleneimine-soaked glass fiber membranes. After washing, the radioactivity captured on the membranes was measured by liquid scintillation.

Alternatively, 0.1 μM *cis*-[^{14}C]UCA or 0.1 μM [^{14}C]5-HT was precipitated with 5 μg/ml anti-*cis*-UCA or 5 μg/ml anti-5-HT antibody in the presence or absence of 10^{-6} to 10^{-9} M nonradioactive ligand. After 3 h at 37°, the receptor–ligand complexes were captured on 3% polyethyleneimine-soaked glass fiber filters, and the signal captured on the filters was measured by liquid scintillation.

To determine the affinity of *cis*-UCA binding to the 5-HT_{2A} receptor, saturation binding curves were constructed. Briefly, 2×10^5 L-NGC-5-HT_{2A} cells were plated in 96-well tissue culture dishes the evening before the experiment. On the day of the experiment, the medium was removed and various dilutions of *cis*-[^{3}H]UCA (0.001–6 nM in cRPMI medium 1640) were added to the confluent monolayers and incubated for 3 h in the cold. After incubation, the cells were washed three times with ice-cold PBS and then lysed with 1 M NaOH. The radioactivity in the washes and in the cell lysate was determined by liquid scintillation counting. Nonspecific binding was determined by measuring binding of the radiolabeled ligand to LM(TK^-) cells, which are devoid of 5-HT receptors and do not bind serotonin (51). The saturation binding curve, K_d, and B_{max} were determined by using Prism Statistical Software (GraphPad, San Diego, CA).

Intracellular Calcium Flux. LM(TK^-) and L-NGC-5-HT_{2A} cells, plated on 22 × 30-mm glass coverslips, were loaded with 10 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 1 h at 37°C. The coverslips were washed thoroughly with PBS and then mounted on a 1.5-ml volume chamber and bathed in Hanks' balanced salt solution (HBSS) with Ca^{2+} at room temperature. The chamber was placed on an epifluorescence/phase-contrast microscope for Ca^{2+} imaging and quantitation. After a baseline $[Ca^{2+}]_i$ was established, cells were then treated with 10 μM serotonin and/or *cis*-UCA in the presence or absence of ketanserin. An INCA workstation (Intracellular Imaging, Cincinnati, OH) was used to quantify $[Ca^{2+}]_i$ levels based on fura-2 fluorescence. Fluorescence was monitored using a ×20 fluorescence objective. Cells were illuminated alternately at excitation wavelengths of 340 and 380 nm by using a xenon arc lamp. The emitted fluorescence was monitored at 511 nm with a video camera, and the calculated free $[Ca^{2+}]_i$ was determined using the cell-free calibration curve. Calcium concentrations were determined using a cell-free calibration curve and INCA software (Win 3.1 version; Intracellular Imaging), as described previously (52). Results are given as mean ± SD from at least 10–20 cells.

DTH. The dorsal hair of C3H/HeNCr mice (National Cancer Institute, Frederick, MD) was removed with electric clippers, and the mice were exposed to 10 kJ/m^2 of UVB radiation from sunlamps (FS 40; National Biological). Alternatively, 5 μmol *cis*-UCA or 250 pmol serotonin were injected into the peritoneal cavity. Anti-*cis*-UCA antibody or antiserotonin antibody (5 μg of protein per mouse, i.p.) was given 30 min before UV exposure or *cis*-UCA injection. Similarly, serotonin receptor antagonists (500 nmol per mouse) were injected 30 min before UV exposure or *cis*-UCA or serotonin injection. Five days later, the animals were immunized by injecting 10^7 formalin-fixed *C. albicans* into each flank. Nine days later, the mice were challenged with antigen, and the immune response was measured 18–24 h later. The data are expressed as the mean change in footpad swelling ± SD ($n = 5$). Statistical differences between the controls and experimental groups were determined by one-way ANOVA, followed by the Dunnett's multiple comparison test. Probabilities <0.05 were considered significant (Prism Statistical Software; GraphPad). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

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