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Mechanisms of permanent loss of olfactory receptor neurons induced by the herbicide 2,6-dichlorobenzonitrile: effects on stem cells and noninvolvement of acute induction of the inflammatory cytokine IL-6

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Abstract

We explored the mechanisms underlying the differential effects of two olfactory toxicants, the herbicide 2,6-dichlorobenzonitrile (DCBN) and the anti-thyroid drug methimazole (MMZ), on olfactory receptor neuron (ORN) regeneration in mouse olfactory epithelium (OE). DCBN, but not MMZ, induced inflammation-like pathological changes in OE, and DCBN increased interleukin IL-6 levels in nasal-wash fluid to much greater magnitude and duration than did MMZ. At 24 h after DCBN injection, the population of horizontal basal cells (HBCs; reserve, normally quiescent OE stem cells) lining the DMM became severely depleted as some of them detached from the basal lamina, and sloughed into the nasal cavity along with the globose basal cells (GBCs; heterogeneous population of stem and progenitor cells), neurons, and sustentacular cells of the neuroepithelium. In contrast, the layer of HBCs remained intact in MMZ-treated mice, as only the mature elements of the neuroepithelium were shed. Despite the respiratory metaplasia accompanying the greater severity of the DCBN lesion, residual HBCs that survived intoxication were activated by the injury and contributed to the metaplastic respiratory epithelium, as shown by tracing their descendants in a K5CreEr^{T2}:^{fl}(stop)TdTomato strain of mice in which recombination causes HBCs to express TdTomato in advance of the lesion. But, contrary to published observations with MMZ, the HBCs failed to form ORNs. A role for IL-6 in suppressing ORN regeneration in DCBN-treated mice was rejected by the failure of the anti-inflammatory drug dexamethasone to prevent the subsequent respiratory metaplasia in the DMM, suggesting that other factors lead to HBC neuro-incompetence.

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Keywords

Dichlobenil; methimazole; olfactory receptor neurons; inflammatory cytokines; horizontal basal cells; globose basal cells; respiratory metaplasia; neurodegeneration; regeneration

Introduction

The herbicide 2,6-dichlorobenzonitrile (DCBN, also named dichlobenil) is one of the most potent olfactory toxicants in rodents (Brandt et al., 1990). It differs from many other olfactory toxicants, such as methimazole (MMZ, 1-methyl-2-mercaptoimidazole, an antithyroid drug) (Brittebo, 1995; Genter et al., 1995), by its ability to induce permanent loss of olfactory receptor neurons (ORNs) in exposed animals. The differential effects of DCBN and MMZ on ORN regeneration in mice was first reported by the Brittebo group (Bergman et al., 2002). In their study, 3 months after DCBN treatment (at 25 mg/kg, i.p., twice), the dorsomedial part of olfactory region was covered by respiratory-like epithelium (i.e., respiratory metaplasia), following the initial acute injury; whereas, 3 months after MMZ treatment (at 50 mg/kg, i.p., twice), the olfactory neuroepithelium was restored following the initial acute injury.

The mechanisms of olfactory toxicity of DCBN and MMZ have been explored in a number of studies. Both compounds require P450-mediated bioactivation in the target tissue for their tissue-specific toxicity in the olfactory mucosa (Brittebo, 1995; 1997), and the olfactory mucosa-predominant P450 isoform CYP2A5 is at least partially involved in their metabolic activation in vivo (Xie et al., 2010; 2011). Both compounds form protein adducts in vivo: strong radioactive signals representing covalent adducts were detected in the olfactory mucosa by whole-body autoradiography following intravenous injection of radiolabeled DCBN or MMZ to mice at a dose of 5 mg/kg (Brandt et al., 1990; Bergman and Brittebo, 1999). Both compounds induce dose-dependent decreases in the levels of nonprotein sulfhydryl residues (mainly glutathione, GSH), as well as detachment of neuroepithelium and tissue damage in the lamina propria (Brittebo, 1995; Bergstrom et al., 2003). A notable difference, however, is that while MMZ induces cell death in the OE primarily through apoptosis (Sakamoto et al., 2007), which is associated with minimal inflammatory response, DCBN-induced cell death in the OE was accompanied by excessive nasal inflammation and necrosis (Brandt et al., 1990; Bergman et al., 2002; Franzén et al., 2006; Mancuso et al., 1997). Nevertheless, the precise mechanism of the differential effects of the two olfactory toxicants on ORN regeneration, including the role of nasal inflammation, and the involvement of olfactory stem cells, remains unclear.

In the present study, we have tested the hypotheses that the differing abilities of DCBN and MMZ to induce inflammatory responses in the nasal cavity play a role in the differential effects of the two toxicants on ORN regeneration, and that the two compounds differ in their effects on olfactory epithelial stem cells (i.e., globose basal cells [GBCs] and horizontal basal cells [HBCs]) in the damaged region. We first confirmed the differential ability of DCBN and MMZ to induce nasal inflammation and respiratory metaplasia in the dorsomedial region of the nasal cavity in the C57BL/6 (B6) mice. We then quantified the levels of four representative inflammatory cytokines [interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ] in mouse nasal-wash fluid after DCBN or MMZ treatment. We also monitored the changes in HBCs in the dorsomedial meatus (DMM, the part that undergoes respiratory metaplasia following DCBN treatment) at various times following DCBN or MMZ treatment. We assessed the contributions of spared HBCs to the metaplastic tissue, by defining the progeny of the HBCs in the epithelium that forms in response to DCBN in a K5CreEr^{T2}:fl(stop)TdTomato strain of mice, in which

Cre-mediated recombination causes HBCs to express TdTomato in advance of the lesion. We further tested the ability of the antiinflammatory steroid dexamethasone (DEX) to prevent the permanent loss of ORNs induced by DCBN.

Our results showed for the first time that DCBN exposure leads to prolonged increases in nasal IL-6 levels, loss of dorsomedial GBCs and some HBCs, and failure of the residual and repopulating basal cells to differentiate into ORNs. Our findings indicate that the differing ability of DCBN and MMZ to induce permanent loss of ORNs is due at least partly to their differential effects on OE stem cells in the damaged region. However, we also find that the acute surge in IL-6 production following DCBN treatment is unlikely responsible for DCBN-induced HBC detachment or the subsequent failure to regenerate the ORNs. These results provide novel insights on mechanisms of olfactory toxicity of xenobiotic compounds, and on the fate of olfactory stem cells upon chemical-induced tissue injury.

Materials and Methods

Chemicals and animal treatments

DCBN (97% pure), MMZ (>99% pure), DEX, and other chemicals were purchased from Sigma Aldrich (St. Louis, MO), unless stated otherwise. B6 mice were from breeding stocks maintained at the Wadsworth Center. Animals were maintained at 22°C with a 12-h on, 12-h off light cycle and were allowed free access to water and a standard laboratory chow. Mice were treated with a single intraperitoneal dose of DCBN or MMZ. DCBN was dissolved in a mixture (1:4; v/v) of dimethyl sulfoxide (DMSO; from Calbiochem) and corn oil, and administered at 5 µl/g body weight. MMZ was dissolved in pure, sterile water at 10 mg/ml. DEX was prepared as a suspension in sterile saline, at 2.5 mg/ml; the preparation was mixed vigorously using a vortex immediately prior to administration via subcutaneous injection. Animals were dosed at 10:00-11:00 AM, local time.

A bigenic strain of transgenic mice was used to trace the descendants of HBCs that remained after DCBN lesion. Toward this end, B6 mice bearing a K5CreER^{T2} transgene (a gift of Pierre Chambon, CNR, Strasbourg, France) were bred to B6 mice bearing the targeted insertion of a floxed(stop)CAG-TdTomato allele into the ROSA26 locus (strain AI9, JAX#007909). Five-week old mice were treated i.p. with 300 mg/kg tamoxifen suspended in corn oil. Three weeks later, bigenic mice were treated i.p. with DCBN, as above, and then survived for an additional eight weeks before euthanasia and tissue harvesting.

All animal use protocols were approved by either the Wadsworth or the Tufts School of Medicine Committee for the Humane Use of Animals.

Determination of cytokines in nasal-wash fluid

Mouse nasal-wash fluid was collected, after the animals were sacrificed, by flushing the nasal cavity with phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl), essentially as reported (Hirano et al., 2003). Briefly, after removal of the mandible, 0.25 ml of ice-cold PBS was gently injected into the nasal cavity through a posterior opening of the nose, and nasal-wash fluid was collected from the anterior openings of the nose. The collected samples were centrifuged at 1,000g for 10 min at room temperature, and the supernatant was used for cytokine determination. The contents of IL-1, IL-6, TNF- α , and IFN- γ in the nasal-wash samples were analyzed using the multiplex Luminex bead technology according the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, 25-µl aliquots of the nasal-wash fluid were incubated with antibody-coated microparticles (beads) overnight at 4°C. Washed beads were further incubated with biotin-labeled anti-mouse antibodies for 1 h at room temperature, followed by incubation with streptavidin-phycoerythrin for 30 min. Samples were then analyzed using

the Luminex model 100TM instrument (Luminex, Austin, TX) and the LiquiChip Analyzer 1.0 Software (Qiagen). Standard curves, constructed using known concentrations of recombinant mouse cytokines, were used for quantitation. In some experiments, IL-6 was also determined using an ELISA DuoSet kit (R&D Systems); for these experiments, the nasal cavity was washed with 1 ml of PBS, and 100- μ l aliquots of nasalwash fluid were analyzed.

Histopathology and immunohistochemistry

For general histopathological examination, tissue blocks containing the nasal passages were dissected and soaked in Bouin's solution for two weeks, for fixation and decalcification, as described previously (Gu et al., 2005). Each tissue block was cut into smaller blocks at four anterior-rostral levels, as described by Young (Young, 1981). Paraffin sections (4 μ m) from levels 1-4 were stained with hematoxylin and eosin (H&E) for pathological examination. Tissue sectioning and staining were performed at the Wadsworth Center Pathology core facility. For immunohistochemistry, the tissue sections were subjected to antigen retrieval with the citrate solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), and then incubated with a blocking buffer containing goat or donkey serum (Invitrogen, Grand Island, NY) to prevent nonspecific binding. The following primary antibodies were used: goat anti-olfactory marker protein (OMP) (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-paired box 6 (Pax6) protein (1:300, Covance, Emeryville, CA), and rabbit anti-keratin 5 (K5) (1:500, Covance). The secondary antibodies used were Alexa-594-conjugated species-specific IgG (1:200, Invitrogen). Sections were mounted with Prolong mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). All images were obtained using a Nikon (Melville, NY) Eclipse 50i microscope, fitted with a digital camera, at the Wadsworth Center Light Microscopy core.

For the HBC lineage tracing experiment, animals were given an overdose of an anesthetic cocktail consisting of ketamine (37.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.25 mg/kg) after the appropriate survival times. After induction of profound anesthesia, the mice were transcardially perfused with phosphate-buffered 1% paraformaldehyde-lysine-sodium periodate fixative (McLean and Nakane, 1974); subsequently, the tissue was dissected, decalcified by soaking for 48 hours in saturated neutral EDTA, cryoprotected in 30% sucrose, and sectioned with a cryomicrotome. The following primary antibodies were used: rabbit anti-PGP (Ultraclone, 1:1200); rat anti-CD133 (eBioscience, 1: 100); goat anti-CD54 (R&D, 1:100). The secondary antibodies used were Alexa-488-conjugated species-specific IgG (1:200, Invitrogen); after staining the sections were mounted with n-propyl gallate in glycerin-PBS. Sections were imaged with a Spot RT2 color digital camera attached to a Nikon 800E microscope. Images were adjusted for contrast, illumination and balance, but were otherwise unmanipulated.

Other methods

Statistical significance of differences among multiple groups in various parameters was examined using one-way ANOVA on ranks, followed by Dunn's post-hoc test, with use of the SigmaStat software (Systat, San Jose, CA).

Results

Differential ability of DCBN and MMZ to induce respiratory metaplasia in the dorsomedial region of the nasal cavity

Previous studies of the abilities of DCBN and MMZ to induce respiratory metaplasia were conducted on NMRI mice (Bergman et al., 2002). Our earlier studies on the acute toxicity of DCBN and MMZ in the nasal mucosa were conducted on B6 mice (Xie et al., 2010; 2011).

In this study, we first confirmed the toxicant dose and time course required to demonstrate the unique ability of DCBN to induce respiratory metaplasia in the B6 mice. As shown in Figure 1, eight weeks after a single intraperitoneal injection of DCBN (at 50 mg/kg), the dorsal medial meatus (DMM) of mouse nasal cavity displayed typical respiratory metaplasia, i.e., it was covered by a respiratory-like epithelium consisting of a single layer of nuclei (Fig. 1A, right panel), instead of the typical pseudostratified OE with multiple layers of nuclei found in vehicle-treated control mice (Fig. 1A, left panel). The permanent loss of ORNs was confirmed by the absence of immunostaining for olfactory marker protein (OMP), a well-established marker for mature ORN (Monti-Graziadei et al., 1977), in the DMM of DCBN-treated mice at eight weeks following DCBN treatment (Fig. 1B, right panel). As controls, OMP was detected abundantly in the OE and olfactory nerve bundles in the submucosa of vehicle-treated control mice (Fig. 1B, middle panel), but no signal was detected when the primary antibody was omitted (Fig. 1B, right panel). Additional studies (Fig. 1C, middle panel) indicated that, even at a much reduced DCBN dose of 12.5 mg/kg, the damaged OE still failed to recover by four weeks after DCBN injection; the DMM region was still covered by non-neural epithelium.

In contrast to DCBN, treatment with MMZ did not cause permanent changes to the OE, at either 50 mg/kg (not shown) or 75 mg/kg (Fig. 1C). The OE was already recovered at four weeks after the initial treatment with MMZ; the DMM region was covered by normal pseudostratified neuroepithelium in both MMZ-treated and vehicle-treated mice. As a control, both DCBN and MMZ treatments were found to cause much of the thickness of the OE in the DMM region to slough into the nasal cavity, when examined at 24 h (Fig. 2) or 48 h (not shown) after a single injection. Therefore, the intact OE seen at four weeks after MMZ treatment represents newly recovered neuroepithelium. The vehicle-treated groups (for either DCBN or MMZ) were not different from untreated mice at the various time points analyzed (not shown).

Differential ability of DCBN and MMZ to induce loss of HBCs in the dorsomedial OE

Histological analysis of the OE at 24 h after a single injection with DCBN or MMZ revealed large differences between the two toxicants in the extent of the acute pathological changes in the nasal cavity (Fig. 2). Early signs of nasal inflammation, including an edematous lamina propria, as well as a proteinaceous exudate and cell debris in the nasal cavity were observed in DCBN-treated mice, with apparent loss of most basal cells and breaching of the basement membrane. In contrast, the damage to the epithelium after MMZ treatment led to the exfoliation of mature neurons and sustentacular cells with the split between sloughed and retained cells located at or immediately apical to the layer of basal cells leaving behind a near complete layer of HBCs (arrow; Fig. 2) and a sparse collection of other cells, presumably GBCs, superficial to them; the basal lamina remained intact, no exudate formed in the nasal cavity and no overt signs of nasal inflammation were observed. The apparent loss of most basal cells in the DCBN-treated mice was further confirmed, by using an antibody against K5, a marker for horizontal basal cells (HBCs), one type of stem cell in the OE (Leung et al., 2007). As shown in Figure 3, a continuous layer of K5-positive cells (HBCs) is superficial to and attached to the basal lamina, and interrupted only occasionally by the duct of Bowman's glands (indicated by *) (Fig. 3A). At 24 h after DCBN injection, many of the K5-positive HBCs in the dorsomedial OE slough into the nasal cavity along with the more superficial cells, which would necessarily include GBCs (indicated by the blue DAPI stain for nuclei) (Fig. 3B). In contrast, the HBCs were intact in MMZ-treated mice, despite the shedding of the more superficial layers (Fig. 3C). Notably, at the doses tested, neither DCBN nor MMZ induced HBC detachment in regions other than the DMM (Fig. 3D-3F).

The time course of recovery, with particular emphasis on the basal cell compartment, was examined in DCBN-treated mice, in order to gauge whether (and when) the basal cells return to the damaged area. As shown in Figure 4, two days after DCBN injection, there was little or no K5 staining in the dorsal medial OE. However, as early as four days after DCBN injection, a mere 48 hours later, the DMM has reconstituted a basal lamina and a population, indeed thicker than normal, of nascent K5-positive cells. By eight weeks after DCBN injection, an epithelium has been reconstituted and lines the DMM, but is now composed of a single layer of ciliated columnar respiratory-like epithelial cells that supersedes a layer of K5-positive cells as well as some cyst-like structures in the lamina propria that also include a basal layer of K5-positive cells.

To confirm further the identity of the regenerated K5-positive cells in DCBN-treated mice, we stained the tissue sections with an antibody to Pax6, which is a transcription factor that is expressed by HBCs among other cell types in the normal OE (Nomura et al., 2007; Guo et al., 2010). In keeping with previously published descriptions, Pax6 immunoreactivity localized mainly to the nuclei of supporting cells (near the apical surface) and basal cells, in vehicle-treated mice (Fig. 5). At 24 h after DCBN treatment, Pax-6 staining near the basement membrane largely disappeared, while Pax-6 positive nuclei are visible among the sloughed OE cells. The repopulated epithelial cells seen at 4 and 7 days after DCBN treatment were weakly positive for Pax6; but the nascent basal, epithelial, as well as glandular-like cells seen at 8 weeks after DCBN were strongly positive for Pax6 staining. Overall, the time-course of changes in Pax6 staining in the epithelial cells after DCBN treatment was consistent with the changes in K5 staining.

HBCs that persist after DCBN contribute to metaplastic respiratory epithelium

The degree of disruption in the OE caused by DCBN makes it difficult to assess the cell type(s) responsible for generating the metaplastic respiratory epithelium that emerges quickly after the initial damage. In addition, HBCs quickly lose their expression of K5 under certain circumstances, as observed following MeBr lesion in the ventrolateral OE of rats (Schwob et al., 1995). In order to assess the contribution of spared HBCs to the metaplastic tissue, a lineage tracing approach was used to define the progeny of the HBCs, to the epithelium that forms in response to DCBN. Bigenic mice were bred to encode a conventional transgene consisting of the K5 promoter driving the mutated recombinase CreER^{T2} as well as the floxed (stop)-CAG-TdTomato construct inserted in the ROSA26 locus (Materials and Methods). Systemic administration of tamoxifen causes nuclear translocation of the recombinase in K5-expressing HBCs, deletion of the stop cassette, and expression of TdTomato. The K5-CreER^{T2} transgenic mouse is the same line, originally made by the Chambon lab, that was used in other experiments designed to accomplish HBC-limited recombination, and other lineage tracing experiments have also utilized this transgene in concert with reporter constructs inserted into the ROSA locus (Leung et al, 2007). In other experiments we have demonstrated that recombination is essentially nil in the absence of tamoxifen (hence leakiness is not an issue).

These bigenic mice were administered tamoxifen three weeks in advance of lesion, to insure that the drug will have disappeared from the animal by the time of DCBN injection, and then euthanized eight weeks following lesion. TdTomato-positive cells are present and abundant in the metaplastic respiratory epithelium lining the DMM of the DCBN-lesioned animals (Fig. 6A, B and inset). In some portions of the lining of the DMM, the TdTomato-positive cells form an uninterrupted band of columnar epithelial cells, which, on close examination, are seen to be capped by cilia, which stain for the presence of CD133 (prominin-1) (Fig 6A, B) and the beta-4 form of tubulin (not shown). CD133 and beta-4-tubulin are expressed on cilia and are characteristic of the normal respiratory epithelial lining of the anteroventral nose (Fig. 6C and inset) (Corbeil et al., 2013; Krolewski et al., 2012). An abrupt fall-off in

CD133 staining clearly marks the boundary between metaplastic respiratory epithelium and neuron-containing olfactory epithelium (arrow in Fig. 6A, abrupt disappearance of PGP9.5-positive olfactory neurons in Fig. 6E). In other areas, the HBC progeny are more scattered, but are, nonetheless, ciliated columnar epithelial cells, a cell type that is not observed in normal olfactory epithelium. In addition to the columnar epithelial cells, TdTomato-positive basal cells are also seen in the metaplastic respiratory epithelium and stain for CD54 (ICAM), a marker that is characteristic of K5-positive basal cells of normal respiratory epithelium as well as HBCs.

Differential ability of DCBN and MMZ to induce nasal IL-6 secretion

The apparent difference between DCBN and MMZ in their abilities to induce nasal inflammation (as shown in Fig. 2) was further explored by an analysis of the levels of four commonly occurring inflammatory cytokines, IL-1, IL-6, TNF-, and IFN-, in nasal-wash fluid. Among the four cytokines, only IL-6 and IFN- were detected, with detection limits of 20 pg/ml and 2.4 pg/ml for IL-1 and TNF-, respectively (data not shown). The levels of IFN- in the nasal-wash samples did not show any significant change after DCBN or MMZ treatment (Fig. 6A); in contrast, the IL-6 levels were increased after both treatments (Fig. 6B). IL-6 levels in the nasal-wash fluid increased ~10 fold at as early as 6 h after DCBN treatment, and ~100 fold ($P < 0.01$) at 24 h after DCBN treatment, compared to levels in nasal-wash fluid from vehicle-treated mice. For comparison, IL-6 levels in the nasal-wash fluid were also increased at 6 h after MMZ treatment, by ~30 fold ($P < 0.05$), compared to levels in nasal-wash fluid from vehicle-treated mice. However, this increase was not sustained over time; at 24 h after MMZ injection, the IL-6 levels were no longer significantly higher than in vehicle-treated mice. There was no difference in the basal IL-6 levels among the various vehicle-treated groups, at 2, 6, or 24 h after the treatment. Thus, DCBN and MMZ differed in the extent and time course of induction of IL-6 secretion into the nasal cavity.

Effects of DEX treatment on DCBN-induced changes

To test the potential role of IL-6 in mediating DCBN's enhanced toxicity relative to MMZ, we determined whether DEX, an anti-inflammatory agent, can block DCBN-induced increase in nasal IL-6 secretion, as well as DCBN-induced loss of HBCs and the subsequent respiratory metaplasia in the dorsomedial region of the nasal cavity. As shown in Figure 7B, DEX treatments (5 mg/kg, given s.c. at 0 and 2 h after an intraperitoneal injection of DCBN) almost completely inhibited the IL-6 increase seen at 24 h after mice were injected with DCBN alone. However, the same DEX treatment paradigm did not protect the OE from DCBN-induced metaplasia. In mice treated with both DCBN and DEX, the dorsal medial region of the nasal cavity was covered by a single layer of nascent epithelial cells, with no Bowman's glands, but with bone formation, in the lamina propria four weeks after DCBN injection, as was observed in mice treated with DCBN and saline (Fig. 8A). Likewise, the same DEX treatment paradigm also did not protect the DMM against DCBN-induced HBC depletion. As shown in Figure 8B, at 24 h after DCBN injection, the K5-positive basal cell layer was mainly detached from the submucosa in mice treated with both DCBN and DEX, as it was in mice treated with DCBN and saline. Thus, these results indicate that DCBN-induced acute increase in nasal IL-6 levels did not play a critical role in DCBN-induced HBC loss or the subsequent respiratory metaplasia in the olfactory mucosa.

Discussion

Successful regeneration in the OE as a neuroepithelium requires the availability of intact olfactory neural stem cells as well as the presence of appropriate tissue environment, which is likely to include cellular and molecular cues regulating cellular differentiation during the

recovery after severe injury (Jang et al., 2003). Disruption of these factors can result in failure of reconstituting a population of GBCs, olfactory neurons, sustentacular cells and the other components of the OE. Substantial evidence suggests that stem cells in the OE reside among the basal cells, which include the HBCs and the globose basal cells (Beites et al., 2005). Several recent studies (Leung et al., 2007; Iwai et al., 2008; Fletcher et al., 2011) provide evidence that HBCs function as a kind of neural stem cell in the OE, at least under environmental conditions that permit recovery of olfactory epithelium; these cells have been shown to replenish ORNs robustly after direct epithelial injury (Leung et al., 2007), and to a much more limited extent in the context of an epithelium where neuronal turnover is low or accelerated by the destruction of the olfactory bulb (Leung et al., 2007; Iwai et al., 2008). That the GBCs are also, and perhaps the more upstream, kind of stem cell is also supported by substantial evidence. One piece of evidence is the ability of the GBCs to produce all olfactory epithelial cell types following transplantation from a purely neurogenic environment (where such pluripotency is not playing out), which contrasts with the failure of HBCs under similar conditions (Goldstein et al., 1998; Chen et al., 2004). Another piece of evidence is the derivation of HBCs from GBCs during development (Packard et al., 2011).

The regeneration of the OE via the proliferation of stem cells and differentiation of their progeny takes place in a homeostatic environment that involves numerous signaling factors, including various cytokines (Beites et al., 2005). The ability of cytokines to influence the regeneration of the ORNs was recently demonstrated in a study of a novel TNF- transgenic mouse model, in which TNF- expression (and consequent inflammation) can be induced specifically in the OE (Lane et al., 2010). In that mouse model, the TNF- induced inflammation remarkably compromised the regeneration of ORNs, although the effect was not permanent. Additionally, neutrophilic rhinitis, induced in mice by intranasal instillation of roridin A (a macrocyclic trichothecene mycotoxin), was found to be associated with significant increases in the levels of several inflammatory cytokines, including TNF- and IL-6, in the nasal lavage, as well as atrophy of OE and loss of ORNs (Corps et al., 2010).

In the present study, we showed that the DCBN-induced permanent loss of ORNs in the DMM was associated with a loss of olfactory stem cells (both GBCs and HBCs) as well as changes in the homeostasis of the stem cell niche (e.g., prolonged increase in IL-6 production). Based on our observation of the ability of DCBN, but not MMZ, to induce inflammation-like features in the olfactory mucosa, and on the widely held view that inflammatory cytokines, especially IL-6, have inhibitory effects on the differentiation of neural stem cells (Taga and Fukuda, 2005; also reviewed by Mathieu et al., 2010), we thought that the inflammatory cytokines released in the olfactory mucosa after DCBN treatment, especially IL-6, would play a critical role in the failure of the ORN regeneration. In that regard, although increases in IL-6 levels were observed following treatment of the animals by either DCBN or MMZ, the magnitude as well as duration of the response were much greater following DCBN treatment (100-fold maximal increase, lasting more than 24 h) than following MMZ treatment (30-fold maximal increase, lasting less than 24 h). However, such a role by IL-6 (or other cytokines that may respond to DEX treatment) in suppressing ORN regeneration in DCBN-treated mice was ruled out by the failure of the DEX treatment to prevent DCBN-induced respiratory metaplasia in the DMM, despite the inhibition of the acute surge in nasal IL-6 production by DEX.

Notably, of the four cytokines analyzed, IL-6 was the only one that showed a significant increase after DCBN or MMZ treatment. This observation was consistent with an earlier report that IL-6 is the most sensitive to ORN injuries among commonly occurring inflammatory cytokines analyzed (IL-1, IL-1, IL-6, TNF-, and macrophage inflammatory protein-2) (Islam et al., 2007). It has also been shown that expression of IL-6

and leukemia inhibitory factor (LIF), which is another IL-6 family cytokine, as well as their receptors, was up-regulated in the olfactory mucosa after apoptosis of ORNs induced by olfactory bulb ablation (Nan et al., 2001).

The differential effects of DCBN and MMZ on olfactory basal cells in the acute toxicity phase are quite clearcut: the extensive sloughing of HBCs and, necessarily, the GBCs superficial to them and destruction of the basal lamina with exudation into the nasal cavity occurred only in DCBN-treated mice, but not in MMZ-treated mice. Notably, our study is the first to report an easily accomplished scenario whereby all GBCs and most HBCs are lost after chemical-induced toxicity; previous studies on ORN regeneration in the OE, either under normal conditions or after tissue injuries, were conducted under conditions that preserved a substantial population of GBCs and HBCs (Schwob et al., 1995; Leung et al., 2007; Iwai et al., 2008). In our study, the DCBN-damaged region was soon repopulated with what appear to be new K5-positive/Pax6-positive basal cells and CD133-positive/beta-4 tubulin-positive ciliated columnar epithelial cells. Both of these cell types of the metaplastic respiratory epithelium were generated in substantial numbers from HBCs that were demonstrably in place in the DMM before intoxication, since they were labeled by TdTomato, of which expression was activated by HBC-specific genetic recombination well in advance of the lesion. Thus, the failure to regenerate a neuroepithelium is not a consequence of the complete destruction of HBCs, but more likely the elimination of cues that normally act upon HBCs to push them in a neuronal, olfactory direction, as opposed to the respiratory differentiation that occurs after DCBN. The nature of the cues is not clear, although the more extensive disruption of the basal layer and what we presume to be the complete elimination of GBCs may be responsible.

It remains to be determined why DCBN produced a more severe lesion in the DMM than MMZ. We have found that inhibition of DCBN-induced IL-6 production by DEX did not ameliorate the severity nor prevent the respiratory metaplasia, which suggested that, although DCBN was much more potent than MMZ was in inducing IL-6 production in the olfactory mucosa, IL-6 was not responsible for the HBC detachment. This conclusion is consistent with the recent report that OE inflammation induced loss of mature ORNs and suppressed ORNs regeneration, but did not damage basal cells (Lane et al., 2010). Both DCBN and MMZ appear to initiate their olfactory toxicities in the Bowman's glands in the lamina propria (Mancuso et al., 1997; Burgstrom et al., 2003). As we observed in the present study, in the DMM of DCBN-treated (but not MMZ-treated) mice, most Bowman's glands and axon bundles had disappeared from the lamina propria, replaced by fibrosis and bone formation. We speculate that the drastic changes induced by DCBN treatment in Bowman's glands and the extracellular environment of the basal cells might disrupt the basement membrane, leading to the detachment and sloughing of most HBCs during the acute phase of tissue injury. Similarly, the changed extracellular environment in DCBN-treated mice might also have altered the interactions between the basal lamina and the regenerated basal cells, causing the latter to differentiate into non-neuronal cells (Carter et al., 2004).

The olfactory toxicity of DCBN is presumably mediated through its reactive epoxide intermediates (Ding et al., 1996); whereas the toxicity of MMZ is believed to be mediated through sulfenic and sulfinic acids (Henderson et al., 2004). These respective reactive metabolites differ in chemical properties, such as charge, hydrophobicity, and reactivity, and may therefore attack different protein targets and/or proteins in different subcellular compartments (such as mitochondria and endoplasmic reticulum) or tissue regions (such as epithelium and submucosa), in the olfactory mucosa. Thus, it would be worthwhile to identify proteins that are uniquely adducted in the DMM of DCBN-treated mice, as compared to MMZ-treated mice; these proteins may include those that are crucial for HBC survival, or for their attachment to the basement membrane. Notably, the sensitivity of the

DMM to chemical toxicity was previously associated with the absence of epoxide hydrolase expression in this region of the nasal mucosa in rats (Genter et al., 1995). The selective damage caused by DCBN in the mouse DMM suggests that this region in the mouse also lacks epoxide hydrolase, which can detoxify DCBN epoxides (Ding et al., 1996).

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Abbreviations

DCBN	2,6-dichlorobenzonitrile (or dichlobenil)
MMZ	methimazole
ORNs	olfactory receptor neurons
OE	olfactory epithelium
DMM	dorsal medial meatus
HBCs	horizontal basal cells
DEX	dexamethasone
K5	keratin 5
PBS	phosphate-buffered saline
IL	interleukin
TNF	tumor necrosis factor
IFN	interferon
OMP	olfactory marker protein

Highlights

- The herbicide dichlobenil (DCBN) can damage olfactory epithelium stem cells
- Another olfactory toxicant, methimazole, leaves the olfactory stem cells intact
- DCBN, but not methimazole, induces a prolonged increase in nasal IL-6 levels
- Dexamethasone inhibits DCBN-induced IL-6 production, but not the stem cell loss

Fig. 1. Regeneration of the OE after DCBN or MMZ treatment

Two- to three-month old male and female C57BL/6 mice were treated with a single i.p. injection of DCBN or MMZ, or with the vehicle alone. Representative cross sections of the nasal cavity, obtained at the level 3 of Young (1981), are shown. A, H&E staining. Eight weeks after DCBN injection (at 50 mg/kg), the DMM of the nasal cavity was covered by a single layer of non-neuronal epithelium, in contrast to the pseudostratified neuroepithelium seen in vehicle-treated mice. B, Immunohistochemical staining of the ORN marker, OMP. Eight weeks after DCBN injection, there were no OMP-positive cells in the DMM of the nasal cavity, in contrast to the robust staining of ORNs in the neuroepithelium, and the nerve bundles in the submucosa, in vehicle-treated mice. C, H&E staining. Four weeks after DCBN injection at a lower dose of 12.5 mg/kg, the DMM was still covered by a single layer of non-neuronal epithelium. In contrast, four weeks after MMZ treatment, at 75 mg/kg, the DMM was already covered by normal pseudostratified neuroepithelium. Scale bar: 20 μ m. BG, Bowman's gland; OE, olfactory epithelium; NB, neural bundles. Magnification: 400 $\times$.

Fig. 2. Histological analysis of the acute nasal mucosal damages caused by DCBN or MMZ treatment

Two- to three-month old male C57BL/6 mice were treated with a single i.p. injection of DCBN (at 50 mg/kg), or MMZ (at 50 mg/kg), or with the vehicle alone. Representative H&E stained cross sections of the nasal cavity, obtained at the level 3 of Young (1981), are shown. Twenty four hours after DCBN injection, the nasal cavity was congested by detached epithelium and exudations. In the submucosa, there was edema and degenerated Bowman's glands. In contrast, 24 h after MMZ injection, the detached epithelium was no longer visible, and the submucosa was denuded. *Arrow* indicates the position of the basement membrane. Boxed areas in the top panels are shown at higher magnification in the bottom panels.

Fig. 3. Immunohistochemical staining for K5 at 24 h after DCBN or MMZ injection
Two-to three-month old male C57BL/6 mice were treated with a single i.p. injection of DCBN (at 50 mg/kg) (A,D) or MMZ (at 50 mg/kg) (B,E), or with the vehicle alone (C,F). Representative cross sections of the nasal cavity (DMM, A-C; septum, D-F), obtained at the level 3 of Young (1981), are shown. Nuclei were identified by DAPI staining (blue). K5 was stained as red. *, opening of a Bowman's gland duct; SP, nasal septum; ET, ethmoid turbinate. Magnification: 200 $\times$.

Fig. 4. Time course for changes in K5-positive cells in OE following DCBN treatment

Two- to three-month old male mice were treated with a single i.p. injection of DCBN (50 mg/kg) or MMZ (50 mg/kg), or with the vehicle alone. Tissues were obtained for immunohistochemical staining for K5, at 24 h, or 2, 4, and 7 d, or 8 weeks after DCBN injection. Representative cross sections of the nasal cavity, in the DMM region, obtained at the level 3 of Young (1981), are shown. A negative control (omitting primary antibody) was also included. Arrow indicates position of the basement membrane. Magnification: 400 $\times$.

Fig. 5. Time course for changes in Pax6-positive cells in OE following DCBN treatment

Two- to three-month old male mice were treated with a single i.p. injection of DCBN (50 mg/kg) or MMZ (50 mg/kg), or with the vehicle alone. Tissues were obtained for immunohistochemical staining for Pax6, at 24 h, or 2, 4, and 7 d, or 8 weeks after DCBN injection. Representative cross sections of the nasal cavity, in the DMM region, obtained at the level 3 of Young (1981), are shown. A negative control (omitting primary antibody) was also included. Arrow indicates position of the basement membrane.

Fig. 6. Spared HBCs give rise to metaplastic respiratory epithelium

Bigenic, K5-CreER^{T2}:fl(stop)CAG-TdTomato mice, were administered tamoxifen 3 weeks before DBCN intoxication and then euthanized 8 weeks later. A) The progeny of spared HBCs, marked by the expression of TdTomato are found in the metaplastic respiratory epithelium lining the dorsomedial meatus (dorsal is toward the top of the figure). The boundary between respiratory and olfactory epithelium is marked by the fall-off in CD133 staining as indicated by the arrows. RM – metaplastic respiratory epithelium, OE – olfactory epithelium. B) The HBC-derived cells in the metaplastic respiratory epithelium lining the dorsomedial meatus are both columnar and basal (the three arrows demarcate a row of basal cells. The columnar cells are capped by cilia (inset). C) The basal cell-derived cells in normal, unlesioned respiratory epithelium located anterior and ventral to the olfactory epithelium closely resemble those in the metaplastic respiratory epithelium (inset). D) The HBC-derived columnar cells in the metaplastic respiratory epithelium lining the dorsomedial meatus do not retain CD54 staining, which is characteristic of HBCs, while the basal cells do (arrow). E) The metaplastic respiratory epithelium lining the dorsomedial meatus is sharply bounded, and that boundary is marked by the rapid transition from neuron-containing epithelium in which ORNs are marked by the expression of PGP9.5 to the metaplastic respiratory epithelium characterized by columnar TdTomato expressing epithelial cells. Scale bars: A – 100 μ m; D – 50 μ m (applies to B, C, as well); E - 25 μ m.

Fig. 7. IFN- γ and IL-6 levels in mouse nasal wash fluid after DCBN or MMZ treatment

Two- to three-month old male mice were treated with a single i.p. injection of DCBN (50 mg/kg) or MMZ (50 mg/kg), or with a vehicle. Nasal wash fluid was collected for analysis at 2, 6, or 24 h after the injection, as indicated. For the DEX+DCBN group, mice were treated twice with DEX (s.c, 5 mg/kg, at 0 and 2 h after DCBN injection), and nasal wash fluid was collected for cytokine determination at 24 h after the DCBN injection. IFN- (A) and IL-6 (B) levels are shown by box plots, which show the minimum/maximum, the 25th/75th percentiles, and the median. *, $P < 0.05$; **, $P < 0.01$; compared to the vehicle-treated group ($n=4$; one way ANOVA on ranks, followed by Dunn's post-hoc test).

Fig. 8. Effects of DEX treatment on nasal mucosal damages caused by DCBN treatment

Two- to three-month old male mice were treated with a single i.p. injection of DCBN (at 50 mg/kg) and two subcutaneous injections of saline or DEX (5 mg/kg) at 0 and 2 h after DCBN injection. A, Tissues were obtained for histopathological examination at 4 weeks after DCBN injection. Representative H&E-stained cross sections of the nasal cavity, obtained at the level 3 of Young (1981), are shown. In DCBN-treated groups, metaplastic bone formation was observed in the submucosa. B, Immunohistochemical staining for K5 at 24 h after DCBN. Arrows indicate position of the basement membrane.