Vitamin D3 Deficiency Increases Sinus Mucosa Dendritic Cells in Pediatric Chronic Rhinosinusitis with Nasal Polyps


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What is This?
Vitamin D₃ Deficiency Increases Sinus Mucosa Dendritic Cells in Pediatric Chronic Rhinosinusitis with Nasal Polyps

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. Dendritic cells are professional antigen presenting cells, capable of initiating Th1 or Th2 responses, and have been implicated in the pathogenesis of a number of diseases, including sinusitis. Vitamin D₃ is a steroid hormone that acts on dendritic cells in a manner similar to corticosteroids. Investigators examined whether children with allergic fungal rhinosinusitis (AFRS) or chronic rhinosinusitis with nasal polyposis (CRSwNP) were vitamin D₃ deficient and the relationship of vitamin D₃ deficiency to dendritic cell infiltrate in the sinus mucosa.

Setting. Tertiary care university hospital.

Study Design. Retrospective, controlled study using samples collected from pediatric patients seen from August 2009 to July 2011.

Subjects and Methods. Plasma levels of 25-hydroxy vitamin D₃ were measured by enzyme-linked immunosorbent assay in children (≤ 18 years old) with AFRS, CRSwNP, or CRS without nasal polyposis (CRSsNP) and in controls undergoing surgery for adenotonsillar hypertrophy. Vitamin D₃ levels were confirmed using clinical diagnostic methods for those with CRSwNP or AFRS. Tissue samples were immunohistochemically stained for the dendritic cell marker CD209 and the costimulatory molecules CD80 and CD86.

Results. There was no difference in mean vitamin D₃ levels between control and CRSsNP, whereas mean CRSwNP and AFRS levels were both well below the minimum recommended level of 30 ng/mL and significantly lower than control and CRSsNP levels. CD209⁺ dendritic cells inversely correlated with vitamin D₃ but not costimulatory molecule expression.

Conclusions. These studies identify that children with CRSwNP or AFRS are vitamin D₃ deficient, which may be linked to increased dendritic cell infiltrate. These results suggest a role for vitamin D₃ as a key player in the immunopathology of pediatric CRSwNP.

Keywords

vitamin D, allergic fungal rhinosinusitis, dendritic cell, pediatric chronic rhinosinusitis

Chronic rhinosinusitis (CRS) encompasses a wide variety of clinical and immunological phenotypes. Chronic rhinosinusitis without nasal polyposis (CRSsNP) has elevated levels of both Th1 and Th2 mediators, resulting in a mixed neutrophilic, eosinophilic infiltrate.¹⁻³ Chronic rhinosinusitis with nasal polyposis (CRSwNP), which includes nonatopic and atopic forms, displays a Th2 skewed immune phenotype including elevated numbers of mast cells and eosinophils that occur in similar levels in nonatopic and atopic forms.⁴ Allergic fungal rhinosinusitis (AFRS), an atopic form subset of CRSwNP, also displays a Th2 skewed immune phenotype. The clinical

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significance of this up-regulation of Th2 cytokines is that they can further drive many of the physical symptoms associated with CRS, including stimulation of rhinorrhea and mucus production. The mechanism driving the observed Th2 skewing in CRSwNP and AFRS is largely unknown.

Whereas the subsets of CRS are different, so are the characteristics of adult versus pediatric CRS. For example, whereas 58% of adults with CRS report rhinorrhea, this occurs in more than 80% of children. Adults also experience a greater degree of thickening in the epithelium and basement membrane than do children with CRS. Differences in immune infiltrate exist between adult and pediatric CRS. A study comparing the immunological differences in adult and pediatric CRS demonstrated that compared with adult CRS, pediatric CRS is characterized by a lower number of eosinophils and higher numbers of neutrophils, lymphocytes, and monocytes/macrophages. One cell population not examined in these studies was dendritic cells (DCs).

Dendritic cells play a significant role in a number of human respiratory diseases including chronic obstructive pulmonary disease, allergic rhinitis, and asthma. Dendritic cells are highly efficient antigen presenting cells capable of directing T-cell responses toward either a Th1 or a Th2 response. The ability of DCs to regulate Th1/Th2 skewing implicates them as potential mediators of the Th2 skewing observed in CRS. Previous studies by our laboratory have shown that adults with CRSwNP or AFRS have increased numbers of DCs compared with controls or subjects with CRSsNP. However, it is unknown whether pediatric CRS displays a similar elevation in DC infiltrate or costimulatory molecule expression.

One potent regulator of DCs that increasing evidence suggests plays an important role in respiratory health is the steroid hormone, vitamin D3 (VD3). The mechanism by which VD3 regulates the immune system is similar to that of many corticosteroids. For example, VD3 plays an important role as an immune regulator through its ability to block monocyte to DC differentiation and maturation, similar to that of corticosteroids. Furthermore, in its active form VD3 increases DC interleukin-10 production, thereby diminishing DC stimulation of Th1/Th2 differentiation, resulting in a more tolerogenic state. Active 1,25VD3 also recruits interleukin-10, producing T-regulatory (Treg) cells, which could help reduce inflammation. Several studies have shown that VD3 can block chemokine synthesis and immune cell recruitment.

Globally, the frequency of VD3 deficiency is increasing, due to a number of factors including obesity, increased time indoors, and increased sunscreen use. Studying the upper airway, Pinto et al. observed that African Americans with allergic rhinitis have lower VD3 levels than race- andagematched controls. Studies by our laboratory have shown that adults with CRSwNP or AFRS are VD3 deficient and have levels significantly lower than CRSsNP or control. Children with asthma, allergies, and/or CRS may be at increased risk of developing VD3 deficiencies because many are encouraged to limit their exposure to outdoor allergens, thus reducing their exposure to the sun. Therefore, in these retrospective studies we examined whether children with CRS are more likely to be VD3 deficient and whether this could affect local DC infiltrate.

Methods

Clinical Evaluation

Studies were conducted retrospectively at the Medical University of South Carolina and were conducted with approval of the Institutional Review Board for Human Research at the Medical University of South Carolina. All patients were 18 years of age or less at time of surgery or clinic visit. Patients were divided among 4 diagnostic groups: control, CRSsNP, CRSwNP, and AFRS. Control patients were undergoing repair of spontaneous cerebrospinal fluid leak, juvenile nasopharyngeal angiofibroma, and adenotonsillar hypertrophy (blood collection only) and had no history of sinusitis and no radiographic or endoscopic evidence of inflammatory sinus disease at time of surgery. CRSsNP patients were diagnosed through clinical and radiographic examinations that revealed inflammatory sinus disease without frank nasal polypsis and no subjective history of atopy. AFRS patients met the classic Bent and Kuhn criteria. Patients who had taken oral steroids or immunotherapy within 30 days of surgery or clinic visit were excluded from these studies.

Determination of VD3 Deficiency

Levels of 25-OH VD3 were determined from blood draw at 2 different time points. Plasma from control, CRSsNP, CRSwNP, and AFRS patients undergoing endoscopic sinus surgery was collected at the time of surgery, and levels were measured by enzyme-linked immunosorbent assay (ELISA; Alpco Immunoassays, Salem, New Hampshire) according to the manufacturer’s instructions. To validate the results we had obtained by ELISA in a different cohort of patients, VD3 insufficiency was measured on consecutive patients as part of their standard of care during clinic visits for patients with CRSwNP or AFRS. In these patients, 25-OH VD3 levels were measured by liquid chromatography/tandem mass spectrometry by the Medical University of South Carolina’s Department of Laboratory Services.

Immunohistochemistry

Biopsy specimens collected during surgery were placed in Tissue-Tek O.C.T. medium (Sakura Finetek, Torrance, California), snap-frozen in liquid nitrogen, and stored at −80°C until use. A Leica CM 1850 cryostat (Wetzlar, Germany) was used to cut tissue specimens (8-μm thickness), which were then affixed to Vectabond (Vector Labs, Burlingame, California) coated slides. The sections were frozen and stored at −20°C until processing.

Tissue specimens from control, CRSsNP, CRSwNP, and AFRS groups were fixed using acetone for 6 minutes at room temperature. Slides were then subjected to 10%
hydrogen peroxide for 2 hours to quench endogenous peroxidase activity. Tissue specimens were treated with 5% swine serum for 20 minutes to block nonspecific antibody staining and subsequently were incubated with commercially available antibodies. We examined the myeloid DC marker CD209 (DC-SIGN) as well as CD80 and CD86, costimulatory molecules expressed by DCs. Primary antibodies or isotype controls were incubated for 1 hour at the following concentrations: CD80 (1:20), CD86 (1:20), or CD209 (1:50) (Abcam, Cambridge, Massachusetts). Following this, slides were subjected to biotinylated secondary antibody (Dako, Carpinteria, California) for a period of 25 minutes. Next, streptavidin–horseradish peroxidase (Dako) was added to the slides and allowed to incubate for 25 minutes. The specimens were then subjected to NovaRED (Vector Labs) substrate solution for 5 minutes, after which the tissue was counterstained with hematoxylin (Dako). As this was a retrospective study, for some patients there was not adequate tissue to complete all 3 stains on every patient. 

**Table 1. Patient Demographics and Sample Information for Samples Collected at Time of Surgery**

<table>
<thead>
<tr>
<th>Patient ID</th>
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<th>Age, y</th>
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<th>Gender</th>
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<td>AA</td>
<td>M</td>
<td>CD80, CD86, CD209</td>
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<tr>
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<td>M</td>
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<td>Control</td>
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<td>5</td>
<td>C</td>
<td>F</td>
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<td>C</td>
<td>F</td>
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The photographer was blinded to patient diagnosis and took 3 random photomicrographs of each slide at ×40 magnification. The number of positive staining cells was counted by 3 graders blinded to diagnostic category and then averaged to yield a mean number of positive cells per high-powered field. The photomicrographs shown are representative results of multiple patients in the indicated cohort.

**Statistical Analysis**

Statistical analysis was conducted using GraphPad Prism 5.02 software (La Jolla, California). Values were first determined to follow a normal distribution using a D’Agostino & Pearson omnibus normality test. A 1-way analysis of variance (ANOVA) with post hoc unpaired Student *t* test was used to determine statistically significant differences between patient cohorts’VD3 levels. A nonparametric 2-tailed was used for histological analyses. Two-way ANOVA was conducted to determine whether differences observed in VD3 levels were influenced by gender, race, or asthmatic status. For analysis in which plasma and sinus tissue were available from the same patient, a Pearson correlation analysis was used to determine whether there was a correlation between a patient’s own VD3 levels and sinus immune infiltrate.
Results

DC Infiltrate Is Increased in CRSwNP and AFRS, Whereas Costimulatory Molecule Expression Is Increased in All Forms of Pediatric CRS

In these studies, we examined DC infiltrate in the sinus mucosa of children with CRSsNP, CRSwNP, or AFRS (Figure 1). Although control patients demonstrated little staining for any of the 3 DC markers, statistically significant increases in staining were observed among the various groups (ANOVA; CD209 \( P = .003 \), CD80 \( P = .01 \), and CD86 \( P = .01 \)). CRSsNP CD209 staining was not increased compared with control (\( P = .326 \)) (Figure 2a). In contrast, compared with control or CRSsNP, CD209 expression was significantly elevated in CRSwNP and AFRS (\( P < .01 \) and \( P = .001 \), respectively). No statistically significant difference was observed in CD209 cells between CRSwNP and AFRS (\( P = .21 \)). Expression of the CD80 and CD86 was significantly elevated in CRSsNP, CRSwNP, and AFRS compared with controls (\( P = .001 \) and \( P < .05 \), \( P = .001 \), \( P = .001 \), respectively) (Figure 2b, c). The results demonstrate that DC numbers are elevated in the sinus mucosa of CRSwNP and AFRS and that costimulatory molecule expression is increased in all forms of pediatric CRS compared with controls.

Children With CRSwNP Are VD3 Deficient, Whereas Those with AFRS Are Insufficient

Next we examined whether children with CRSwNP or AFRS were VD3 deficient. In children whose blood was drawn at the time of surgery, control and CRSsNP both demonstrated similar, sufficient levels of VD3 of 38.4 ± 3.2 and 36.3 ± 3.5 ng/mL, respectively, but those levels were significantly different than the levels of VD3 with CRSwNP or AFRS (ANOVA \( P = .001 \)) (Figure 3). Mean CRSwNP levels were insufficient at 22.1 ± 3.2 ng/mL and were significantly lower than control or CRSsNP (\( P < .05 \) compared with either group). Levels of AFRS 25-OH VD3 were observed to be insufficient at 17.7 ± 4.5 ng/mL and were significantly lower than control (\( P = .0018 \)) or CRSsNP (\( P = .0044 \)).

To validate the results we obtained in our laboratory, we examined VD3 levels in children with CRSwNP or AFRS seen in clinic by sending serum for testing to the Medical University of South Carolina’s clinical laboratory. This allowed us to confirm that the results we obtained by ELISA could be reproduced using diagnostic methods. Similar to the results we obtained by ELISA, children seen in clinic were VD3 insufficient with CRSwNP 25-OH VD3 levels reported at 23.1 ± 8.6 and AFRS 17.6 ± 8.1 ng/mL (Figure 4a). Next, we examined other cofactors that could be influencing alterations in VD3 levels, including gender, race, asthma, and age (Figure 4b-e). Because of the limited number of patients, CRSwNP and AFRS were combined for 2-way ANOVA analysis of gender, race, and asthmatic status. This analysis demonstrated that changes in VD3 status were independent of gender (Figure 4b) (\( P = .5233 \)) or race (Figure 4c) (\( P = .0511 \)). Asthmatic status did not influence VD3 levels (\( P = .2831 \)) in CRSwNP and AFRS (4 patients whose asthmatic status was unknown were excluded). Age was examined as a continuum rather than

Figure 1. Immunohistochemical staining for DC infiltrate in the sinus. Representative staining shown for isotype control (a-d), CD209 (e-h), CD80 (i-l), and CD86 (m-p). Examples of positive staining are identified by arrows in the respective photomicrograph. AFRS, allergic fungal rhinosinusitis; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps.
using arbitrary cutoff, with a regression analysis revealing that in this population age did not correlate with VD₃ levels ($R^2 = 0.053$, $P = .2994$).

**Vitamin D₃ Levels Inversely Correlate with Sinus Mucosa Numbers of DCs but Not Costimulatory Molecule Expression**

After determining that children with CRSwNP and AFRS were VD₃ deficient, we next examined whether there was an association between VD₃ and elevated number of DCs in the sinus mucosa. To this end, we used patients who had both plasma and tissue collected at the time of surgery (similar to the results in Figures 1 and 2). Figure 5a demonstrates that a strong inverse correlation exists between number of CD209⁺ DCs and 25-OH VD₃ status ($R^2 = 0.7198$, $P = .01$). However, sinus expression of neither CD80 (Figure 5b) nor CD86 (Figure 5c) correlated with VD₃ levels ($P = .717$ and $P = .5594$, respectively). These results demonstrate that VD₃ deficiency is associated with increased numbers of DCs in the sinus mucosa of CRS but not costimulatory molecule expression in pediatric CRSwNP or AFRS.

**Discussion**

Although many similarities exist between adult and pediatric CRS, previous studies have reported differing immune profiles. We have previously reported increased numbers of DCs and DC costimulatory molecule expression in adult CRSwNP and AFRS compared with controls or CRSsNP.¹³,²⁷ However, because of other reports showing differences between adult and pediatric CRS immune infiltrate, we were unsure whether our findings about DC infiltrate in adults would apply to pediatric CRS. Similar to what we observed in adults, DC numbers were elevated in children with CRSwNP and AFRS. This would be expected, as both CRSwNP and AFRS possess a Th2 skewed profile, which in lower airway diseases such as asthma has been linked to increased DC infiltrate.²⁸-³⁰ However, unlike our adult studies, the current pediatric study revealed an increase in CD80 and CD86 expression in pediatric CRSsNP compared with control. Furthermore, in adults the highest levels of CD80 and CD86 expression were observed in patients with AFRS, whereas in children CRSwNP displayed the most staining. One hypothesis for this observed

**Figure 2.** Quantification of immunohistochemistry staining. Quantitative analysis of (a) CD209, (b) CD80, and (c) CD86 cells per field in sinus mucosa of controls and patients with chronic rhinosinusitis without nasal polyps (CRSsNP), chronic rhinosinusitis with nasal polyposis (CRSwNP), and allergic fungal rhinosinusitis (AFRS). *$P < .01$, **$P = .001$ or #$P < .05$ vs control.

**Figure 3.** Plasma levels of 25-OH vitamin D₃ by enzyme-linked immunosorbent assay. Points represent results of individual patients. *$P < .05$ vs control or CRSsNP. **$P < .004$ vs control or CRSsNP. AFRS, allergic fungal rhinosinusitis; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyposis.

**Figure 5a** demonstrates that a strong inverse correlation exists between number of CD209⁺ DCs and 25-OH VD₃ status ($R^2 = 0.7198$, $P = .01$). However, sinus expression of neither CD80 (Figure 5b) nor CD86 (Figure 5c) correlated with VD₃ levels ($P = .717$ and $P = .5594$, respectively). These results demonstrate that VD₃ deficiency is associated with increased numbers of DCs in the sinus mucosa of CRS but not costimulatory molecule expression in pediatric CRSwNP or AFRS.
difference is that CD80 and CD86 expression may be occurring on macrophages, which have been shown to be elevated at greater levels in pediatric CRS than in adult CRS. Further evidence for this alternative hypothesis comes from murine and human asthma studies in which CD80 and CD86 are constitutively expressed by alveolar macrophages.

In an attempt to identify a possible mechanism accounting for elevated DC sinus infiltrate in CRSwNP and AFRS, we examined the possible role of VD3, which is a potent regulator of DC maturation and migration. Although it is estimated that 18% of children in the United States have deficiencies in VD3, we observed that more than 90% of children with CRSwNP or AFRS were VD3 deficient. These results paralleled our previous studies in adults with CRS that identified deficiencies in those with CRSwNP or AFRS.

One limitation of our adult VD3 studies is that they focused solely on the relationship between VD3 and circulating DCs. Similar to what we observed in circulation, local CD209+ cell numbers inversely correlated with systemic VD3. These results are consistent with previous in vitro studies demonstrating the ability of VD3 to modulate myeloid DC functions. One surprising finding in these studies is that no correlation exists between VD3 and local CD86 expression. These results were a bit unexpected since

![Figure 4. Vitamin D3 (VD3) analysis by clinical methods and examination of influencing factors. (a) VD3, (b) race, (c) gender, (d) asthmatic status, or (e) age. (•) = VD3 sufficient; (■) = VD3 insufficient. AFRS, allergic fungal rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyposis.](https://example.com/figure4.png)
VD3 has been shown to block DC CD86 expression. However, many of these studies examined the effects of active 1,25-OH VD3 in vitro and not the inactive form measured clinically to diagnose VD3 deficiency and used in these studies. An alternative hypothesis would be that in pediatric CRS, CD86 expression may be VD3 independent. This would be supported by the results that CD86 expression was elevated in CRSsNP, which overall was VD3 sufficient. Another possible explanation for these results is that as mentioned previously, CD86 may be expressed by more heavily on macrophages in pediatric CRS, which, if similar to adults, is independent of VD3. Alternatively, in CRSsNP, healthy VD3 levels may be inducing macrophage formation, as VD3 has been shown to promote monocyte to macrophage differentiation and proliferation. Studies examining polyposis in Asian have found that DC-LAMP is elevated in CRSwNP regardless of whether inflammation is eosinophilic or noneosinophilic. Because of the greater levels of melanin in the skin, persons of Asian descent are at an increased risk of developing VD3 deficiency, with one study suggesting that as many as 94% of Asian adults may be VD3 deficient. This also raises the interesting question, although one that cannot be addressed by this study, as to what role VD3 deficiency may play in driving the immunological changes associated with Asian polyps.

Although these studies identify that children with CRSwNP or AFRS are VD3 deficient, the role of VD3 supplementation remains unclear. In asthmatic patients, who are Th2 skewed similar to CRSwNP, increased 25VD3 was associated with reduced likelihood for hospitalization for asthma-related complications and reduced use of anti-inflammatory medications. Serum 25VD3 levels are also inversely associated with the occurrence of upper respiratory tract infections, and this association was even stronger in those with asthma and chronic obstructive pulmonary disease. However, although numerous studies examining airway diseases have observed that these patients are at a greater risk for VD3 deficiency, few studies have been published showing whether VD3 supplementation results in improvement of clinical or immunological outcomes. In psoriasis, topical VD analogues have been shown to reduce the number of DCs in the skin and promote DCs to induce T-cell tolerance, resulting in reduced contact hypersensitivity responses. One double-blinded study suggested that VD3 supplementation during the winter may reduce the incidence of influenza A. Another example of the possible impact of VD3 supplementation of respiratory healthy is that in patients with chronic obstructive pulmonary disease and severe VD3 deficiency, supplementation reduced the number of exacerbations. In steroid-resistant asthmatic patients it has been shown that VD3 administration can down-regulate Th2 skewing. However, it is unclear whether VD3 supplementation would improve immune dysfunction or provide clinical benefits to children with CRS. Correction of VD3 deficiency may result in clinical improvement because of its anti-inflammatory properties and would offer a novel, safe, cost-effective therapy for these patients.

Figure 5. Correlation between VD3 and sinus immune infiltrate. Pearson correlation analysis demonstrating 25-OH VD3 vs number of (a) CD209⁠⁺, (b) CD80⁠⁺, and (c) CD86⁠⁺ cells in the sinus mucosa.
Acknowledgment

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Author Contributions

Jennifer K. Mulligan, involved in all aspects of research; David R. White, study design, manuscript editing, data interpretation, tissue procurement; Eric W. Wang, study design, manuscript editing, data interpretation, histology scoring; S. Ritter Sansoni, significant involvement in acquisition of data; Helen Moses, significant involvement in acquisition of data; Robert J. Yawn, significant involvement in acquisition of data; Carol Wagner, data analysis including statistic, study design with specific involved in the role of vitamin D3; Sarah E. Casey, histology scoring, data analysis, technical assistant with data acquisition; Ryan M. Mulligan, histology scoring, data analysis, technical assistant with acquisition, manuscript preparation; Rodney J. Schlosser, study design, tissue procurement, manuscript preparation, data analysis and interpretation.

Disclosures

Competing interests: David R. White, Medtronic, Inc, ad hoc consultant (tonsil product, no relationship to this manuscript); Rodney J. Schlosser, BrainLAB, Olympus, Sunovian, NeilMed, consultant/ advisory board; Medtronic, Arthocare, NeilMed, grant support. (These disclosures are not related to this manuscript.)

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