Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis

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Background: Allergic eye diseases are complex inflammatory conditions of the conjunctiva that are becoming increasingly prevalent and present an increasing economic burden because of direct and indirect health expenditures.
Objective: We sought to identify factors that may synergize with antigen-induced allergic inflammation and lead to allergic conjunctivitis. We used a murine model of allergic conjunctivitis to test the effect of oxidative stress generated by pollen oxidases using nicotinamide adenine dinucleotide (reduced) or nicotinamide adenine dinucleotide phosphate (reduced) (NAD[P]H) as an electron donor present in pollen grains.
Methods: Reactive oxygen species (ROS) generation by hydrated *Ambrosia artemisiifolia* pollen (short ragweed pollen; RWP) grains was determined by using 2′-7′-dihydrodichlorofluorescein diacetate, nitroblue tetrazolium reduction, and Amplex Red assay. The RWP-induced changes in intracellular ROS levels were examined in A549 cells, human primary bronchial epithelial cells, and murine conjunctiva.
Results: Ragweed pollen grains contain NAD(P)H oxidase activity, which is diphenyleneiodonium-sensitive and quinacrine-sensitive and sodium azide-resistant. These NAD(P)H oxidases generate a superoxide anion that can be converted to H_2O_2 by pollen grain–associated superoxide dismutase. These diffusible oxygen radicals from pollen grains increase intracellular ROS levels in cultured epithelial cells and murine conjunctiva. Similar phenomena were observed in sensitized and naive mice, indicating that the RWP-induced oxidative stress in conjunctival epithelium is independent of adaptive immunity. Inactivation of NAD(P)H oxidase activity in RWP decreases the immediate-type hypersensitivity and inflammatory cell infiltration into the conjunctiva.
Conclusion: Our data suggest that ROS generated by NAD(P)H oxidases in pollen grains intensify immediate allergic reactions and recruitment of inflammatory cells in murine conjunctiva.
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Key words: *Pollen NAD(P)H oxidase, oxidative stress, epithelium, conjunctivitis*

Seasonal allergic conjunctivitis, or hay fever, one of the most common allergic diseases, results in significant morbidity and presents an increasing economic burden because of direct health expenditures, as well as less evident cost factors, such as lost work time. The immediate hypersensitivity associated with this disease is characterized by allergen-mediated cross-linking of IgE on mast cells, leading to degranulation and release of mediators, including histamine, tryptase, leukotrienes, cytokines, and platelet-activating factors.[1] These mediators stimulate nerve endings, dilate blood vessels, and recruit inflammatory cells to the reaction site, causing clinical symptoms[1] such as itching, erythema, and palpebral and conjunctival edema. The late phase of this disease is associated with an accumulation of inflammatory cells in the conjunctiva.[2]

Antigenic components of pollen grains have been implicated in mediating allergic inflammation. However, recent studies have shown that pollens also release proteolytic enzymes[3] and eicosanoid-like lipid mediators,[4] which may influence the course of allergic reactions. Other evidence suggests that reactive oxygen species (ROS) play a prominent role in the pathogenesis of allergic diseases.[5-9] In allergic rhinitis caused by house dust mites, nasal eosinophils generate H_2O_2, which causes tissue injury and augmentation of the allergic reaction.[10] Another source of ROS is exposure to environmental air pollutants. Ozone, diesel exhaust, and cigarette smoke can generate oxidative stress in the airways and participate in the worsening of disease symptoms.[11-13] An earlier report suggests the existence of a linear relationship between nasal symptoms and ozone levels during periods of high atmospheric pollen in patients with pollen allergy.[14] Ozone exposure augments antigen-induced rhinitis,

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Abbreviations used

H_2DCF-DA: 2′-7′-Dihydro-dichlorofluorescein diacetate
NADH: nicotinamide adenine dinucleotide, reduced
NADPH: nicotinamide adenine dinucleotide phosphate, reduced
NAD(P)H: NADH or NADPH
NBT: Nitroblue tetrazolium
NHBE: Primary normal human bronchial epithelial
$O_2^{\cdot-}$: Superoxide anion
ROS: Reactive oxygen species
RWP: Ragweed pollen
RWP^H: Heat-treated ragweed pollen
SOD: Superoxide dismutase
X+XO: Hypoxanthine and xanthine oxidase

sneezing, nasal secretion, hyperresponsiveness, and eosinophil infiltration in guinea pigs.[15]

Our study reports for the first time that pollen grains contain oxidases using nicotinamide adenine dinucleotide (reduced) or nicotinamide adenine dinucleotide phosphate (reduced) (NAD[P]H) as an electron donor, which produce ROS and lead to oxidative stress in cultured human epithelial cells and murine conjunctiva. Inhibition of NAD(P)H oxidase activity significantly decreased clinical manifestations and late-phase events in a murine model of allergic conjunctivitis. Our data indicate that oxidative stress generated by NAD(P)H oxidase in pollen grains augments immediate-type hypersensitivity reactions and pollen antigen-driven allergic conjunctivitis.

METHODS

Cell cultures and pollen grains

Primary normal human bronchial epithelial (NHBE) cells (catalog #CC-2641) were cultured in BEGM BulletKit medium supplied by the manufacturer (Cambrex Bio Science, Walkersville, Md). The A549 bronchial epithelial cells (American Type Culture Collection, Manassas, Va) were cultured in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL). Pollen grains from weeds (short/common ragweed, *Ambrosia artemisiifolia*; English plantain, *Plantago lanceolata*; redroot pigweed, *Amaranthus retroflexus*; Russian thistle, *Salsola kali)*, grasses (Bermuda grass, *Cynodon dactylon*; redtop, *Agrostis gigantea*; timothy grass, *Phleum pratense*), and trees (white birch, *Betula populifolia*; white oak, *Quercus alba*) were purchased from Greer Laboratory (Lenoir, NC).

Animal sensitization and conjunctival challenge

Female BALB/c mice 6 to 8 weeks old from Harlan Sprague-Dawley (San Diego, Calif) were used for these studies. Mice were sensitized intraperitoneally on days 0 and 4 with 150 μg/mouse ragweed pollen extract (Greer Laboratory) mixed with alum, as previously described.[16] On day 10, conjunctivitis was induced by topical application of 10 μg ragweed pollen (RWP), suspended in 5 μL PBS (pH 7.4), into each eye. Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by UTMB's Animal Care and Use Committee.

Measurement of ROS

Pollen grains were hydrated in PBS for 10 minutes, and then 20 μmol/L 2′-7′-dihydro-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, Ore) was added.[17,18] A change in dichlorofluorescein-mediated fluorescence intensity was assessed in a FLx800 micro plate reader (Bio-Tek Instruments, Winooski, Vt) at 488 nm excitation and 530 nm emission. In parallel experiments, dichlorofluorescein fluorescence was visualized by a NIKON Eclipse TE 200 UV microscope (excitation at 485 nm) (Lewisville, Tex). Images were taken with a Photometrix CoolSNAP Fx digital camera and analyzed with Metamorph software (version 5; Universal Imaging, Downingtown, Pa). NHBE or A549 were cells grown to 70% confluence and loaded with 50 μmol/L H_2DCF-DA at 37°C for 15 minutes. After removing excess probe, the cells were exposed to RWP, heat-treated RWP (RWP^H, 72°C for 30 min), RWP with superoxide dismutase (SOD; 50 U/mL), or RWP pretreated with Tiron (5 mmol/L; Sigma Inc, St Louis, Mo). The change in fluorescence intensity was assessed as described.

The conjunctival epithelia of BALB/c mice were topically loaded with 250 μmol/L carboxy–H_2DCF-DA (Molecular Probes) for 15 minutes. After excess carboxy–H_2DCF-DA was washed out, the conjunctiva of mice were challenged with 5 μL PBS, 100 μg RWP, or 100 μg RWP pretreated with Tiron (10 mmol/L) in 5 μL volume. After 15 minutes, mice were anesthetized, and the eyes were enucleated with the attached lids and intact conjunctiva. Tissues were embedded in optimal cutting temperature medium (Sakura Finetek, Torrance, Calif), frozen, and sectioned. Dichlorofluorescein fluorescence was analyzed as described.

Nitroblue tetrazolium assay

Pollen grains (100 μg/assay) were hydrated in PBS for 10 minutes and mixed with 2 mmol/L nitroblue tetrazolium (NBT) ± nicotinamide adenine dinucleotide (reduced) (NADH) (100 μmol/L) or nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) (100 μmol/L). Mixtures were then incubated for 15 minutes at 37°C. NBT was completely removed by repeated washing steps, and the formazan precipitate was dissolved in methanol.[19] Absorbance was determined at 530 nm (A_{530nm}) on a spectrophotometer (DU 530; Beckman Instruments, Fullerton, Calif).

Measurement of hydrogen peroxide

The H_2O_2 level was measured spectrophotometrically at 560 nm (A_{560nm}) by using an Amplex Red assay kit (Molecular Probes). The reaction mixture (100 μg pollen in 200 μL PBS + 100 μL Amplex Red reagent/horseradish peroxidase solution) was incubated at 37°C for 30 minutes before to the experiment. The H_2O_2 concentrations were calculated by comparison with assays of standard serial dilutions of H_2O_2. The addition of catalase (400 U/mL), but not SOD, decreased A_{560nm} by ~90%.[20]

Clinical evaluation

Twenty minutes after the topical administration of RWP, animals were examined for signs of immediate hypersensitivity, and clinical scores (chemosis, conjunctival redness, lid edema, tearing, and discharge) were determined as previously described.[21]

Histology

The enucleated eyes with the attached lids and intact conjunctiva were immediately fixed in 10% buffered formaldehyde for 24 hours. The tissue was paraffin-embedded, serially sectioned through the central sagittal plane, and stained with Giemsa or hematoxylin and