

Nasal provocation test (NPT) with isolated and associated *Dermatophagoides pteronyssinus* (Dp) and Endotoxin lipopolysaccharide (LPS) in children with allergic rhinitis (AR) and nonallergic controls

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Abstract: *Background:* Inhalation of endotoxin may enhance inflammatory airway response in sensitized asthmatics persons after allergen(s) inhalation.

Objective: To evaluate nasal response to intranasal instillation of *Dermatophagoides pteronyssinus* (Dp), endotoxin (LPS), and to Dp+LPS in children with perennial allergic rhinitis (PAR).

Methods: 10 PAR children (positive skin prick test to Dp) and 10 nonallergic controls (C) undergoing nasal provocation test (NPT), who were quantified by active anterior rhinomanometry and measurement of Total Nasal Resistance (TNR). The NPTs were initially performed with histamine (H; 0.03 to 16.0 mg/mL), and then, at least at weekly intervals, the NPTs were done with Dp (1/100,000 to 1/2.5), LPS (1 to 500 mg/mL) and to Dp+LPS. During NPT with Dp+LPS, Dp concentration was kept constant (1/100,000; 1/10,000; 1/1,000) and was combined with different concentrations of LPS (1, 5, 10, 20 mg/mL). The NPT was considered positive when TNR reached twice the basal TNR.

Results: H and Dp NPTs were positive in all AR children. In group C, H NPT was positive in 60% and Dp NPT was negative in all children. NPT with LPS was positive only in 30% of the AR children. NPT with Dp+LPS was positive in 90% of the AR patients in Dp concentration of 1/1,000 and in LPS concentrations of 5, 10, and 20 mcg/mL. This positive association was observed with Dp concentrations lower than those obtained during NPT with Dp in 60% of AR patients. There were no changes in pulmonary function tests in all children after NPT.

Conclusions: This study suggests that LPS enhances the effects of allergen challenges on nasal airflow. The daily inhalation of allergens plus endotoxin in AR patients does increase the nasal responsiveness.

Key words: allergic rhinitis, mites, lipopolysaccharide, LPS, endotoxin, *D. pteronyssinus*

Introduction

The role of bacteria on dust allergy is not completely understood. Preliminary data suggest that Gram-negative bacteria (GNB) are an immunotoxic component in house dust [1]. The active principle, endotoxin (ET), is responsible for GNB toxic effects and is contained in the bacteria membrane [2]. Chemically, ET is a

lipopolysaccharide (LPS) made up of a hydrophilic heteropolysaccharide (nucleus and specific A chain) linked to a hydrophobic lipid portion, named lipid A, that anchors externally the molecule to the membrane. ETs are thermostable substances, present in organic dust and in the nasal/oral cavity. They are constantly released due to bacterial lysis [3].

LPS interact directly with monocytic cell lineage [4].

They are potent macrophage activators and intensify the synthesis of lipidic inflammatory mediators from arachidonic acid (platelet activating factor, tumoral necrosis factor alpha [TNF- α]) [5-7]. LPS also increase the synthesis of interleukins (IL)-1, IL-6, IL-8, and IL-12 [8,9] and bind to macrophage surface receptors to induce the production of oxygen radicals, nitric oxide [10,11], and lytic enzymes [12].

In vitro studies showed that ET has a potent IL-12 and INF γ synthesis-inducing activity. They are essential cytokines which drive the immune response towards a Th1 profile [8,9,13]. Epidemiological studies have associated early exposition of infants to high levels of ET with lower frequency of allergic sensitization, asthma and atopic diseases, as pointed out by the Hygiene Hypothesis [13,14].

LPS circulate in the blood stream linked to a 60 kDa binding protein named LBP (LPS binding protein) produced in the liver, which enhances LPS inflammatory response [15,16]. The inhalation of LPS (20 μ g) induces (after 45 minutes) a moderate but significant decrease in the forced expiratory volume in the first second (FEV1), in asthmatics rather than in normal individuals [17-19]. LPS also induce a systemic inflammatory response characterized by an increase of α -TNF production, leukocytosis, and neutrophilia [20]. In asthmatic patients, LPS increase eosinophil survival by increasing the production of granulocyte colony-stimulating factor by mononuclear cells [20].

Significant bronchial obstruction after inhalation of LPS is associated to an increase in non-specific hyper-responsiveness, which normalizes in a 24-48-hour period and recovers completely after seven days [17-22].

The inhalation of ET may enhance the inflammatory airway response in sensitized asthmatics after allergen inhalation [22-25]. We studied whether this synergistic action between *Dermatophagoides pteronyssinus* and LPS also occurs in the nasal mucous membrane of children with PAR.

The objectives of this study were to assess the inflammatory effects induced by intranasal LPS instillation, isolated or associated with *D. pteronyssinus*, in PAR children sensitized to *D. pteronyssinus*, and to evaluate the systemic inflammatory effects induced by intranasal instillation of LPS, measured by the number of peripheral leukocytes, neutrophils, and eosinophils.

Patients and Methods

Patients

This study enrolled 10 PAR children (anamnesis and physical examination) aged 6 to 14 years with positive skin prick test (SPT, mean wheal diameter \geq 5mm) [26] to inhaled allergens (*D. pteronyssinus*, mixed mold, cat, dog, and cockroach; IPI-ASAC, Brazil) or sensitized only to *D. pteronyssinus*, and 10 children (aged 6yr 1mo to 13yr

10mo), with personal and familiar negative history of allergic disease, and negative SPT to the same aeroallergens.

Methods

All children were submitted to SPT with *D. pteronyssinus* (112,150 BEU/mL; IPI-ASAC, Brazil) and STP with LPS (*Escherichia coli* O26:B6, Sigma Chemical Co): 1, 5, 10, 20, 100, 200, and 500 mcg/ml. In both SPTs positive (histamine = 10 mg/mL) and negative (saline) controls were used.

NPTs were performed at least at weekly intervals, always in the morning and after acclimatization in a room with controlled temperature (25 to 28°C). NPTs were monitored by active anterior rhinomanometry (AARM; Berger Rhinomanometer S/A) [27,28]. Total nasal resistance (TNR) was measured by calculating the resistance of each nostril separately (P/V): $TNR = \text{RightNR} \times \text{LeftNR} / \text{RightNR} + \text{LeftNR}$, as previously described [29]. NPTs were performed according to the following schedule: a) Day 1 = NPT with Histamine, b) Day 2 = NPT with *D. pteronyssinus*, c) Day 3 = NPT with LPS, d) Day 4 = NPT with LPS plus *D. pteronyssinus* (1:100,000), e) Day 5 = NPT with LPS plus *D. pteronyssinus* (1:10,000), and f) Day 6 = NPT with LPS plus *D. pteronyssinus* (1:1,000).

Histamine and Dp were diluted in 0.9% saline [27,28]. LPS was also diluted weekly in sterile and apyrogenic 0.9% saline solution and stored in apyrogenic tubes at 4 °C until nasal instillation with sterile and apyrogenic pipettes.

NPTs with histamine were performed as follows: after obtaining basal TNR, each nostril was sprayed with 0.1 mL saline, and a new TNR was determined five minutes later (baseline value for NPT). Thereafter, 0.1 ml of histamine at increasing concentrations (0.03; 0.06; 0.12; 0.25; 0.50; 1.0; 2.0; 4.0; 8.0 and 16.0 mg/ml) were instilled in each nostril at 5-minute intervals and a new TNR measurement was obtained [28]. Pulmonary function tests were performed to obtain: FEV1 and forced expiratory flow at 25 - 75% of the forced vital capacity (FEF25-75%).

NPTs with *D. pteronyssinus* were performed in the same way as the NPTs with histamine, except for the TNR measurement intervals, performed 30 minutes after the instillation of the *D. pteronyssinus* concentrations. The doses were: D1 = 1:100,000; D2 = 1:10,000; D3 = 1:1,000; D4 = 1:100; D5 = 1:10; D6 = 1:5 and D7 = 1:2.5, as previously standardized in our lab [28]. The same procedure was accomplished with the NPT with LPS (*Escherichia coli* O26:B6; doses: L1=1; L2=5; L3=10; L4=20; L5=100; L6=200 and L7=500 mcg/mL). A peripheral blood sample was drawn for WBC count before and after 3 hours from the last instillation of LPS.

NPTs were performed as previously described, with LPS (1, 5, 10, and 20 mcg) plus *D. pteronyssinus* (1:100,000); LPS (1, 5, 10, and 20 mcg) plus *D. pteronyssinus* (1:10,000); and LPS (1, 5, 10, and 20 mcg) plus *D. pteronyssinus* (1:1,000).

All patients were challenged with all concentrations (Histamine, Dp, LPS, and Dp+LPS) even when the positive provocative concentration (increase of 100% in baseline TNR) was reached (30,31). A decrease of 20% in basal FEV1 and FEF25-75% values was considered as positive pulmonary response (32).

A subjective assessment of nasal symptoms using a clinical score was performed during all NPTs, as follows: coryza (0 = absent, 1 = mild, 2 = moderate, 3 = severe), sneeze (0 = absent, 1 = 1-5 times, 2 = 5 to 10 times, 3 = more than 10 times), itching (nasal/ocular) (0 = absent, 1 = present), and obstruction (0 = absent/mild, 2 = moderate, 3 = total unilateral or bilateral) [33].

Statistical analysis was performed by Friedman's variance analysis by ranks, Mann-Whitney's test and Wilcoxon's test.

This study was approved by the UNIFESP-EPM Ethical Medical Committee and a signed informed consent was obtained from parents or legal/authorized guardians of all participants.

Results

All patients and 60% of the controls had a positive NPT with histamine. Analyzing the whole allergic group during the NPT with histamine, we observed a significant TNR increase, beginning at the concentration of 2.0 mg/mL. In the control group this increment occurred at the concentration of 8.0 mg/mL (Table 1). Clinical scores increased significantly at lower concentrations of histamine when compared to the increase of TNR, for both groups (Table 1).

NPT with LPS was positive in 40% of the allergic patients and negative in all controls. Studying the whole allergic group during the NPT with LPS, a significant

increase in TNR and in clinical score was found at the concentration of 500.0 mcg/mL (Table 2).

NPTs with Dp were positive only in PAR children. Considering group A as a whole, we observed a significant TNR increase beginning at 1:5.0 concentration and a significant increase of clinical score beginning from 1:100 (Table 3). In group C, we found a significant increase of clinical score beginning at 1/5. The figure shows the concentration of Dp producing this positivity. NPTs with Dp+LPS were positive in 9/10 PAR children. When Dp was associated with LPS, it was equal in 3/9 and lower in 6/9 of them (Figure 1). No concordance was shown among Dp concentrations able to produce a positive SPT and a positive NPT (data not shown).

The number of peripheral neutrophils increased only in the atopic group. No changes in eosinophil numbers were found in controls (Table 4).

During all the NPTs there was no significant reduction in the pulmonary function measurements. SPTs with LPS were negative for all concentrations in all children.

Discussion

ET is considered a modulating factor of clinical manifestations of asthma, and its concentration in house dust has been related to the clinical severity of the disease. Mite-allergic asthmatics have a higher risk of corticosteroid-dependent asthma when exposed to high ET amounts [20]. The role of ET in allergic rhinitis has not been appropriately studied yet.

Although the NPT is well tolerated by children, the concomitant pulmonary function tests limited us to evaluate children older than 6 years, assessing all factors capable to interfere with the results [26,34].

Table 1. Mean total nasal resistances (cm/H₂=/L/s; TNR) and mean clinical scores observed in nasal provocation tests (NPTs) with Histamine. Data from 10 patients with allergic rhinitis and 10 normal controls.

		Histamine (mg/mL)										
TOTAL NASAL RESISTANCE		Basal	0.03	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0
Allergic	0.55	0.57	0.58	0.57	0.59	0.69	0.79	0.91*	1.63*	2.43 ^{+/}	3.63 ^{*/}	
Control	0.40	0.36	0.37	0.37	0.42	0.40	0.45	0.52	0.56	0.59*	0.69*	
CLINICAL SCORE		Basal	0.03	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0
Allergic	0.0	0.7	1.1	1.3	2.1	3.0	3.8*	4.6 ^{*/}	5.9 ^{*/}	7.4 ^{*/}	9.0 ^{*/}	
Control	0.0	0.5	0.3	0.6	0.5	0.9	1.1	1.6	2.0*	2.2*	3.4*	

Friedman's rank test- * p < 0.05 values were significantly higher than basal ones
Mann-Whitney - f p < 0.05 values were significantly higher than controls.

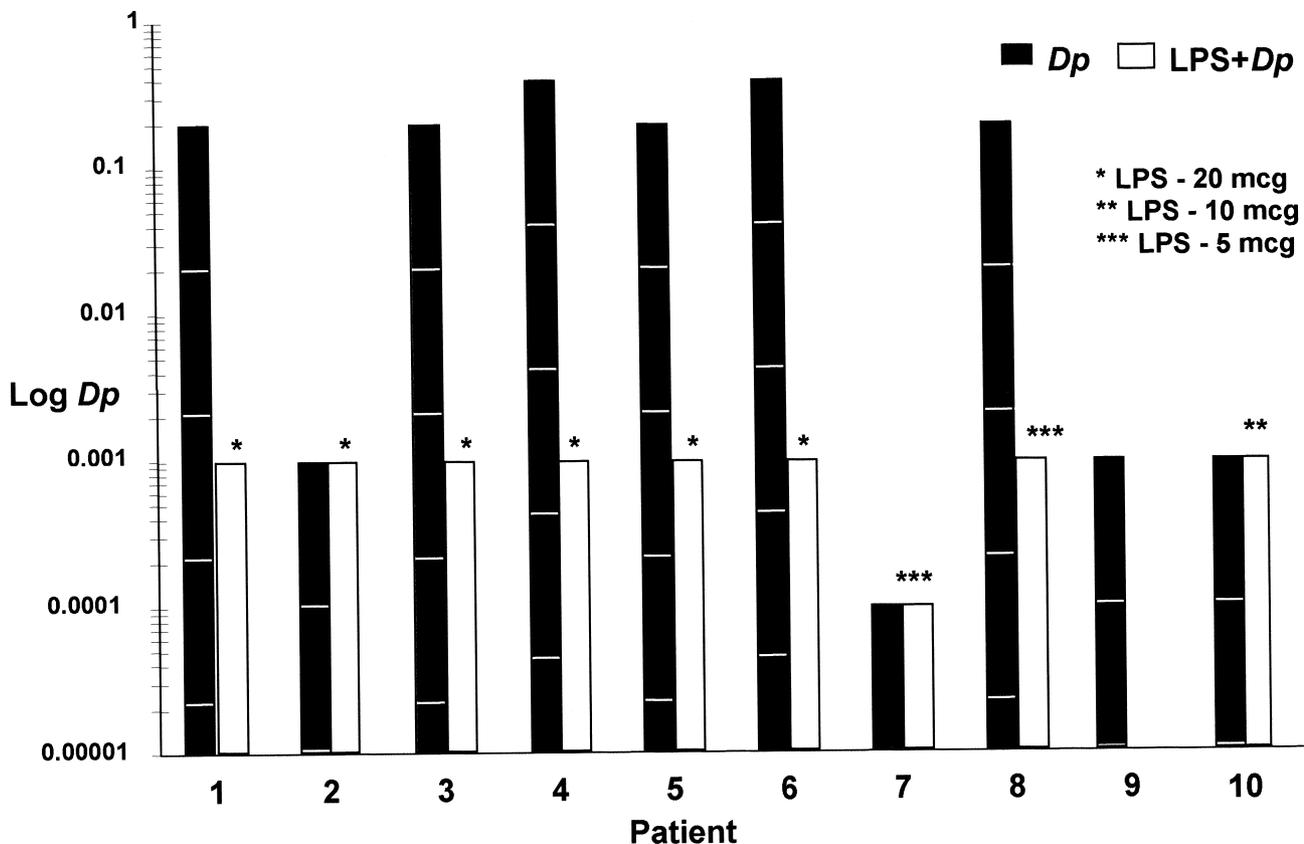


Figure 1. Concentration of *Dermatophagoides pteronyssinus* (Dp) that induces positive nasal provocation test, isolated or associated to lipopolysaccharide (LPS).

Anterior active RNM is a simple and non-invasive method that allows the evaluation of nasal permeability in response to specific and non-specific challenges [35]. As recommended, the volume of provocative solution instilled in NPT must be between 0.1 and 0.2 mL/nosril [31] and all tested solutions were simultaneously applied in both nostrils aiming to avoid misinterpretation due to nasal cycle, and also considering the possibility of reactions due to a reflex of unilateral challenge [36].

Nasal response in NPT was evaluated in various times from 5 to 30 minutes after allergen challenge. We selected 30 minutes because at this point the response to the allergen is greater [31].

NPT with histamine is recognized as an effective tool for diagnosing nasal hyper-reactivity [36]. NPTs with histamine were positive in all patients from group A beginning at concentrations of 2.0 mg/mL. Although 60% of patients from group C showed a NPT positive to histamine, that positivity was found at higher concentrations of histamine (Table 1); this was also true for the clinical score (Table 1). These data are similar to those previously observed by our group [27,28].

All PAR patients were sensitized to *D. pteronyssinus* and were positive for specific NPT. The opposite

occurred within group C (data not shown). The mean TNR and the mean clinical score increased significantly in PAR patients beginning at concentrations of 1/5 and 1/100 concentrations, respectively, and earlier than in patients from group C (Table 2). In previous studies with Dp-allergic patients the positivity seen was 90% during NPT with Dp [28,33].

ET is considered a pro-inflammatory substance able to potentiate inflammatory effects due to specific challenges [36,37]. In this study, LPS doses were the same used in bronchial provocation tests [38], namely, 100, 200 and 500 mcg/mL. However, studies assessing the effects of LPS nasal instillation in TNR are scarce.

Thirty percent of PAR patients were positive for NPT with LPS at the concentration of 500 mcg/mL. All patients from group C were negative for NPT with LPS (data not shown). A significant increase in mean values of TNR and in clinical score was noted in both groups with LPS concentration of 500 mcg/mL (Table 3). SPT with LPS was negative in PAR patients and in all patients from group C (data not shown). The absence of immediate cutaneous response to LPS suggests that the nasal response to LPS might not be dependent on IgE mechanisms in these PAR patients [20,39].

Table 2. Mean total nasal resistances (cm/H₂O/L/s; TNR) and mean clinical scores observed in nasal provocation tests (NPTs) with LPS. Data from 10 patients with allergic rhinitis and 10 normal controls.

		LPS (mcg/mL)						
TOTAL NASAL RESISTANCE								
Group	Basal	1.0	5.0	10.0	20.0	100.0	200.0	500.0
Allergic	0.54	0.46	0.50	0.55	0.59	0.64	0.66	0.92 ^{*/}
Control	0.33	0.26	0.29	0.34	0.38	0.42	0.44	0.49
CLINICAL SCORE								
Allergic	0.0	0.4	0.4	0.3	0.8	0.8	1.4	1.9 [*]
Control	0.0	0.3	0.1	0.2	0.5	0.3	0.4	0.6 [*]

Friedman's rank test - * p < 0.05 values were significantly higher than basal ones
Mann-Whitney - f p < 0.05 values were significantly higher than controls.

After NPT with LPS in PAR patients, a significant increase in neutrophil amount was observed, as well as a reduction in eosinophil amount, but no differences were detected in group C (Table 4), as reported by others [20,40-42]. There were no changes in the number of lymphocytes.

Tucker et al. [43] studying NPT with LPS in atopic patients reported increases in eosinophil, polymorphonuclear cells, and in the granulocyte-macrophage colony-stimulating factor levels (GM-CSF) found in nasal aspirates 4 hours after provocation. No changes were shown for IL-1b, IL-8 and TNF-a levels, suggesting that ET can induce eosinophilic influx to the airways of atopic patients. The data reported by Tucker et al. [43] might explain the reduction in peripheral eosinophils in patients from group A in this study.

To evaluate the LPS enhancing effect on nasal provocation with Dp we carried out NPTs associating both agents. Thus, we selected the 3 lowest concentrations of Dp that produced positive NPT associating them to 4 low concentrations of LPS. The figure shows data from PAR patients, where Dp isolated concentrations alone and those associated with positive NPT-producing LPS were presented. Nine out of 10 patients presented positive Dp+LPS NPT; 6/9 PAR patients presented with a positive NPT with Dp concentration lower than those presented with Dp alone.

From these data, it can be suggested that the nasal reactivity produced by the Dp+LPS association was higher than that produced by isolated Dp or LPS, probably due to an amplification of the allergic inflammatory response. Similar results had already been

Table 3. Mean total nasal resistances (cm/H₂O/L/s; TNR) and mean clinical scores observed in nasal provocation tests (NPT) with *Dermatophagoides pteronyssinus*. Data from 10 patients with allergic rhinitis and 10 normal controls.

		<i>Dermatophagoides pteronyssinus</i>						
TOTAL NASAL RESISTANCE								
Group	Basal	1/100,000	1/10,000	1/1,000	1/100	1/10	1/5	1/2.5
Allergic	0.53	0.58	0.63	0.76	0.91 ^f	1.05 ^f	1.79 ^f	1.82 ^f
Control	0.50	0.47	0.50	0.47	0.43	0.48	0.50	0.70 [*]
CLINICAL SCORE								
Allergic	0.0	1.4	2.1	2.8 ^f	4.6 ^{*,f}	5.9 ^{*,f}	7.5 ^{*,f}	9.0 ^{*,f}
Control	0.0	0.3	0.3	0.5	1.0	1.0	1.9 [*]	2.8 [*]

Friedman's rank test - * p < 0.05 values were significantly higher than basal ones
Mann-Whitney - f p < 0.05 values were significantly higher than controls.

Table 4. Mean absolute number of blood leukocytes, neutrophils and eosinophils (cel/mm³) before and after nasal provocation test with LPS in allergic and control children.

Group	Before			After		
	Leukocytes	Neutrophils	Eosinophils	Leukocytes	Neutrophils	Eosinophils
Allergic	6,850	3,365	541	7,460	4,342*	374
Control	7,420	3,700	298	7,770	3,697	265

Wilcoxon's test - * p < 0.05 higher than before.

documented in atopic asthmatic patients. The analysis of cytokines, mediators and cell influx in nasal lavage of these patients, not performed in this study, might be helpful in clarifying this phenomenon.

Under normal conditions, low levels of LBP, sCD14 and LPS are present in bronchoalveolar lavage (BAL) of asthmatic patients. After allergen challenge, there are changes in the vascular permeability of the lungs, with significant increase of LBP and sCD14 in BAL, so the inhalation of even a small LPS amount may result in an increase of the inflammatory response to local concentrations of LPS [36].

This fact might explain the hypothesis of synergistic action of allergen and LPS inhaled daily and in various concentrations.

No alterations in pulmonary function tests performed after NPT, both in PAR and group C patients were detected.

In conclusion, positive NPT with Dp was observed only among the PAR patients, and 30% of them were positive for NPT with LPS. NPTs with Dp+LPS were positive in 6/9 patients with Dp doses lower than those necessary to promote a positive NPT with Dp. This study suggests that there is a synergism between Dp and LPS in the nasal mucous membrane of atopic individuals. Daily inhalation of allergens combined with ET in PAR patients can raise the nasal responsiveness and probably induce a PAR harder to manage.

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