

T-cell proliferation induced by local application of LPS in the nasal mucosa of nonatopic children

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Background: Recently, there has been a great deal of interest in the role of bacterial products in altering the allergic immune response in early life.

Objective: We sought to develop a model of bacterial inflammation in human subjects to characterize the ex vivo response to LPS in the nasal mucosa of young nonatopic children.

Methods: Using the explant technique, we cultured nasal mucosa in the presence or absence of increasing concentrations of LPS (0.001–1 µg LPS/mL) for 24 hours. Immunocytochemistry and in situ hybridization were used to phenotype the cells and cytokines in the explant, and immunofluorescence was used to study proliferation of T cells in explants cultured with LPS and 5-bromo-2'-deoxyuridine (BrdU).

Results: CD68, CD3, tryptase, elastase, IFN-γ IL-12, and IL-10 immunoreactivity was detected in unstimulated tissue. LPS stimulation caused a dose-dependent increase in the number of CD3⁺ (n = 7, *P* < .05), CD68⁺ (n = 7, *P* < .01), elastase-positive (n = 7, *P* < .01), tryptase-positive (n = 7, *P* < .05), IL-2-positive (n = 6, *P* < .05), IL-12-positive (n = 6, *P* < .05), and IFN-γ-positive (n = 6, *P* < .05) cells compared with media alone. LPS had no effect on major basic protein, IL-4, IL-5, or IL-13 immunoreactivity. LPS (0.1 µg/mL) caused an increase in the number of BrdU-positive cells as early as 2 hours after incubation (n = 8, *P* < .001). The maximal BrdU immunoreactivity was seen 24 hours after incubation with LPS, at which time approximately 70% of BrdU-positive cells were CD3⁺ T cells. **Conclusions:** Our results suggest that LPS is capable of inducing local inflammation, T_H1 cytokine immunoreactivity, and proliferation of CD3⁺ activated T cells in the absence of de novo cell recruitment. This model holds promise for future studies investigating the importance of bacterial products in regulating local immune function in young atopic children. (*J Allergy Clin Immunol* 2002;110:771–6.)

Key words: Immunomodulation, lipopolysaccharide, nasal mucosa, T cell proliferation, children, explants

Abbreviations used

BrdU: 5-Bromo-2'-deoxyuridine

MBP: Major basic protein

RPE: R. Phycoerythrin

TLR4: Toll-like receptor 4

The so-called hygiene hypothesis suggests that decreasing levels of exposure to infections, commensal microbial stimuli in developed countries, or both might be responsible for the increased prevalence of asthma. Experimental evidence suggests that microbial flora, particularly that from the gastrointestinal tract, is the principal trigger for normal postnatal maturation of the immune system during early life.¹ Bacterial LPS, a constituent of the outer membrane of gram-negative bacteria, has been suggested as one potential mediator of these effects. We have previously shown, in an animal model of combined allergic and bacterial inflammation, that exposure of rats to aerosolized LPS during early sensitization protects against their development of IgE and is capable of abolishing allergen-induced inflammation and reduction in lung function.² The mechanisms responsible for this phenomenon remain to be elucidated.

To investigate the mechanisms involved in LPS-induced modification of immune response in early life, we chose to modify the explant technique previously used in our laboratory to study local allergic inflammation.^{3,4} Using explanted biopsy specimens from the nasal mucosa of nonatopic children less than 6 years of age, we studied the effects of in vitro application of LPS on inflammatory cell population and cytokine production. To our knowledge, this is the first time that the role of bacterial LPS has been directly looked at in children at this young age by using the explant system. For this reason, it was important to fully characterize their immune response to LPS at baseline and to find the optimum doses of LPS to be able to correctly interpret the findings of our long-term goal of studying the role of LPS in young atopic children.

In this study we hypothesized that even in the absence of de novo cell recruitment, local application of LPS would lead to a shift toward T_H1 immune phenotype as a result of activation of T_H1-driven inflammatory cells and cytokines. Our results suggest that this explant model might be a useful tool for elucidating mechanisms involved in immunoregulation of the allergic immune response by bacterial products in atopic children.

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METHODS

Subjects

Twelve healthy nonatopic children of both sexes (mean age, 3.6 $\pm$ 0.7 years) were recruited from the Ear, Nose, and Throat Clinic of the Montreal Children's Hospital. These patients had no history of asthma or other allergic diseases and had negative skin prick test responses to a panel of common allergens (including house dust mite, grass pollen, ragweed extract, cat, and dog). Nasal mucosa tissue was obtained from the inferior turbinate (approximately 5 mm in diameter) of children undergoing tonsillectomy, adenoidectomy, nasal septal surgery, or sinus surgery. Informed consent was given by parents or guardians before the procedure, and the study was approved by the Ethics Committee of the McGill University Health Center.

Culture of nasal mucosa explants

Immediately after excision, the biopsy specimen was placed in bicarbonate buffered culture medium and transported on ice to the Meakins-Christie Laboratories for processing. The time between removal of tissue and culture of nasal mucosa was less than 1 hour. Serial sections of tissue were each placed in 30-mm, filtered-well inserts (Millicell CM, Millipore Corp) with a 0.4- μ m pore diameter within a 6-well culture plate (Costar; Corning Inc) containing 2 mL of fresh buffered culture medium. The tissue was oriented with the epithelium being exposed to the air, forming an air-liquid interface to mimic the in vivo situation. Tissue was incubated in the presence or absence of increasing concentrations of LPS (*Escherichia coli* serotype 026:B6, 0.001-1 μ g/mL, Sigma Chemical Co) added directly into the culture medium. The nasal explants were incubated overnight at 37°C in 5% CO₂/95% air.

Fixation and processing

Twenty-four hours after tissue culture, the tissue was fixed for 2 hours at room temperature in 4% paraformaldehyde and then put through a series of 1-hour washes in 15% sucrose/PBS. After the third wash, the tissue was kept overnight at 4°C and blocked the next day in optimal cutting temperature embedding medium (Tissue-Tek Somagen Diagnostics) by means of snap-freezing in isopentane cooled in liquid nitrogen. The tissue was sectioned at 5- μ m thickness onto poly-L-lysine (0.1%)–coated glass slides (Microm HM500, Microm International GmbH) and baked overnight at 37°C.

Immunocytochemistry

Alkaline phosphatase–antialkaline phosphatase immunocytochemistry⁵ was used to identify macrophages (anti-CD68, 1:50, DAKO Diagnostics), pan-T cells (anti-CD3, 1:100, DAKO), activated T cells (anti-CD25, 1:5, DAKO), mast cells (anti-tryptase, 1:250, Cedarlane Laboratories), neutrophils (anti-elastase, 1:100, DAKO), and eosinophils (anti-major basic protein [MBP], 1:30, a gift from Dr R. Moqbel). Monoclonal antibodies against IL-12 (1:20), IFN- γ (1:50), IL-4 (1:50), IL-5 (1:50), and IL-13 (1:25) were used to determine cytokine immunoreactivity in the tissue (R&D Systems). Expression of toll-like receptor 4 (TLR4) was studied by using the polyclonal antibody against TLR4 (1:200, Santa Cruz Biotechnology). Briefly, cryostat sections were incubated with universal blocking solution (DAKO) for 15 minutes at room temperature to block nonspecific binding of antibody. The optimal concentration of each antibody was diluted in standardized diluting buffer (DAKO), and the slides were incubated with primary antibody overnight at 4°C in a humidified chamber. The next day, slides were washed in Tris-buffered saline, incubated with rabbit anti-mouse secondary antibody (DAKO, 1:60) at room temperature for 30 minutes, washed again, and incubated with mouse alkaline phosphatase–antialkaline phosphatase (DAKO, 1:60) at room temperature for 30 minutes. For

the polyclonal TLR4 antibody, the secondary antibody used was biotinylated swine anti-rabbit immunoglobulin (DAKO, 1:300), and Streptavidin-AP (DAKO, 1:200) was used as the tertiary antibody. Immunostaining was developed with Fast Red (Sigma Chemical Company) dissolved in substrate solution (0.5 mmol/L naphthol AS-MX phosphate, 2% dimethylformamide, 1 mmol/L levamisole, and 0.1 mol/L Tris base, pH 8.2). All slides were counterstained with Gill II Haematoxylin, and positive cells appeared red under bright field illumination. For negative controls, the primary antibody was replaced with an isotype-matched control antibody.

In situ hybridization

The sections were hybridized with ³⁵S-labeled antisense. Sense probes coding for human IL-12 and IFN- γ were generated from cDNA and linearized, and in vitro transcription was initiated with the RNA polymerases SP6 and T7 in the presence of ³⁵S-labeled uridine triphosphate to generate radiolabeled antisense (complementary) and sense probes (identical to mRNA) recognizing RNA message encoding IL-12 or IFN- γ . IL-12 and IFN- γ mRNA was identified in the nasal tissue by means of in situ hybridization, as previously described.^{6,7} Briefly, tissue was permeabilized with 0.3% Triton (BDH) and proteinase K (1 μ g/mL, Sigma Chemical Co). Slides were prehybridized in 50% formamide (Sigma) in 2 $\times$ standard sodium citrate solution (BDH) for 15 minutes at 37°C and hybridized with 0.75 $\times$ 10⁶ cpm/slide of radiolabeled probe overnight in a humid chamber at 42°C. Nonspecific binding was removed by means of a series of washes in decreasing concentrations of saline-sodium citrate buffer (4-0.1 $\times$) at 42°C and subsequent treatment with RNase (20 μ g/mL) to destroy excess unbound RNA probe. Slides were dipped in emulsion (Amersham LM-2), stored at 4°C, and developed 2 weeks later in Kodak D19 solution (Eastman Kodak). Slides were counterstained with Gill II Haematoxylin, and cover slips were applied. Positive signal was identified as silver grains over the cells.

Immunofluorescence

Nasal mucosa was incubated with 5-bromo-2'-deoxyuridine (BrdU; 10 μ mol/L, Roche Diagnostics GmbH) in the presence or absence of 0.1 μ g/mL LPS and incubated at 37°C in 5% CO₂/95% air for 2, 6, 12, or 24 hours to investigate the role of LPS in proliferation of T cells ex vivo. After incubation, tissue was processed as described above. The slides were fixed in fixation buffer (70% ethanol and 50 mmol/L glycine, pH 2.0) for 20 minutes at -20°C. Cellular incorporation of BrdU was detected by using an anti-BrdU–specific antibody and visualized with anti-mouse antibody conjugated to fluorescein (BrdU Labeling and Detection Kit, Roche Diagnostics GmbH). T cells were identified by using goat anti-human CD3 antibody conjugated to R. Phycoerythrin (RPE; Cedarlane Laboratories). Briefly, after incubation of sections with universal blocking solution, as described above, slides were incubated with anti-BrdU working solution (1:10, containing nucleases for DNA denaturation) for 30 minutes at 37°C. After the slides were rinsed (3 $\times$ in PBS), the secondary anti-mouse Ig-fluorescein antibody (1:10) was applied for 30 minutes and washed, and goat anti-human CD3-RPE (neat) was applied for 30 minutes at 37°C. The negative controls were performed, as described above, in the presence or absence of goat anti-human CD3-RPE. All slides were covered with PermaFluor mounting medium (Shandon) and evaluated at 660 $\times$ magnification with a fluorescence microscope (LSM410, Carl Zeiss). Proliferating BrdU-positive cells were identified as green, CD3⁺ cells as red, and colocalization of proliferating cells with CD3⁺ T cells as yellow immunostaining.

Quantification

All cells in individual biopsy specimens were counted by 2 observers blindly with an Olympus light microscope (Carson Group Inc) at 400 $\times$ magnification and an eyepiece graticule of 0.202 mm².

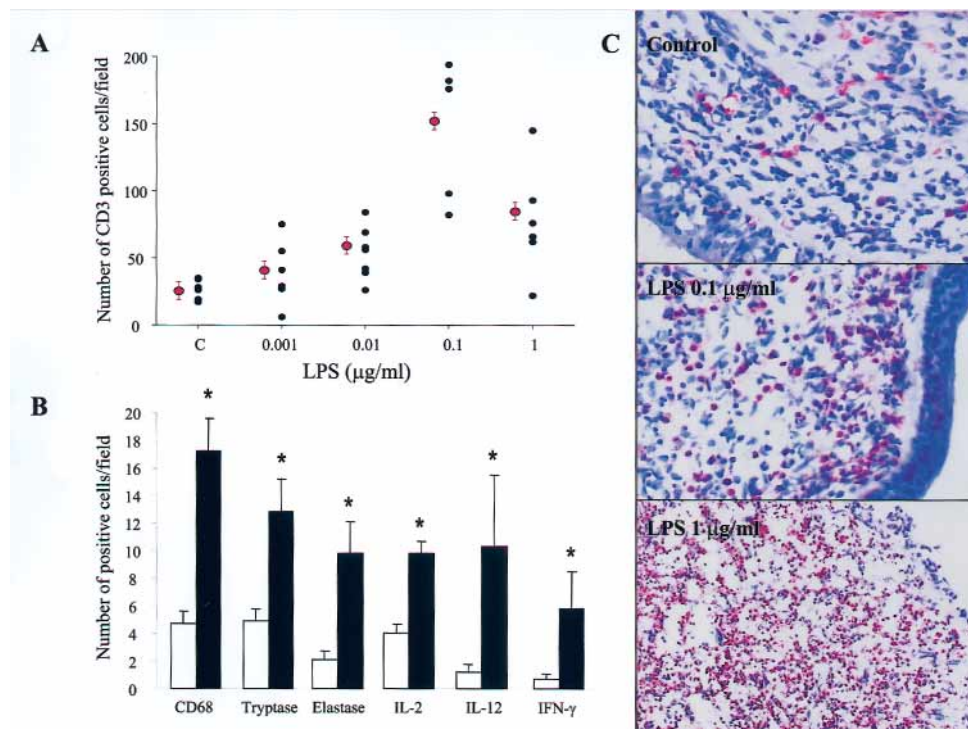


FIG 1. Dose-dependent increase in the number of CD3⁺ T cells in the nasal mucosa after exposure to LPS (0.001–1 µg/mL; **A**) and the cellular (CD68, tryptase, and elastase) and cytokine (IL-2, IL-12, and IFN-γ) profile after stimulation with the optimal concentration of LPS (0.1 µg/mL; **B**). **C** represents an example of CD3 immunoreactivity in unstimulated tissue (*top panel*) after ex vivo stimulation with 0.1 µg/mL LPS (*middle panel*) and 1 µg/mL LPS (*lower panel*). Note that with the highest dose of LPS, epithelial shedding is observed.

In the airway epithelium and subepithelium, the number of positive cells was averaged from 6 to 8 random, nonoverlapping grids and expressed as the mean number of positive cells per field. Immunofluorescence was semiquantitated by using ImageJ software (<http://rsb.info.nih.gov/ij/>) and expressed in optical density units. For slides in which both anti-BrdU and CD3-RPE primary antibodies were used, colocalization was also expressed as the approximate percentage of all BrdU-positive cells that were also CD3⁺.

Statistical analysis

The difference in the number of immunopositive cells for a particular cell type or cytokine in the presence or absence of LPS was analyzed by using ANOVA with Dunnett post hoc analysis for multiple comparisons (Systat version 10, SyStat Inc). Results were expressed as means ± SEM. *P* values of less than .05 were regarded as statistically significant.

RESULTS

Cellular and cytokine profile in unstimulated tissue

Under basal conditions in unstimulated nasal biopsy specimens, CD68, elastase, CD3, IFN-γ, tryptase, IL-2, IL-12, and IL-10 immunoreactivity was detected (Fig 1). In unstimulated tissue the largest proportion of cells present were CD3⁺ T cells, averaging 28.11 ± 2.39 positive cells per field (Fig 1, A), followed by CD68⁺ macrophages (4.67 ± 0.97 per field), tryptase-positive mast cells (4.89 ± 0.80 per field), and elastase-positive

neutrophils (2.11 ± 0.54 per field; Fig 1, B). Baseline expression of IL-2, IL-12, and IFN-γ cytokine immunoreactivity was detected (Fig 1, B) as was TLR4 positivity in unstimulated tissue. No immunoreactivity was detected for MBP (eosinophil marker), IL-4, IL-5, or IL-13.

Cellular and cytokine profile in LPS-stimulated tissue

After LPS stimulation, there was a dose-dependent increase in the number of CD3⁺ cells compared with that seen in media alone ($n = 6$, $P < .05$; Fig 1, A). A similar dose-dependent increase was observed in the number of CD68⁺ ($n = 6$, $P < .05$), tryptase-positive ($n = 7$, $P < .05$), elastase-positive ($n = 6$, $P < .05$), IL-2-positive ($n = 5$, $P < .001$), IL-12-positive ($n = 6$, $P < .05$), and IFN-γ-positive cells ($n = 6$, $P < .05$). At 0.1 µg/mL LPS, the number of CD3⁺ (Fig 1, A), CD68⁺, tryptase-positive, and elastase-positive cells (Fig 1, B) was increased 4.8-, 4.7-, 2.6-, and 3.1-fold, respectively, whereas the number of IL-12-positive and IFN-γ-positive cells was increased almost 9-fold when compared with that seen in unstimulated tissue (Fig 1, B). IL-2 immunoreactivity was increased 2.4-fold in LPS-stimulated tissue (Fig 1, B). LPS (0.1 µg/mL) also significantly increased the number of CD25⁺ cells (activated T cells) from 2.35 ± 0.28 positive cells per field to 11.46 ± 1.87 positive cells per field ($n = 5$, $P < .05$) and significantly increased the number of CD4⁺ T cells ($n = 7$,

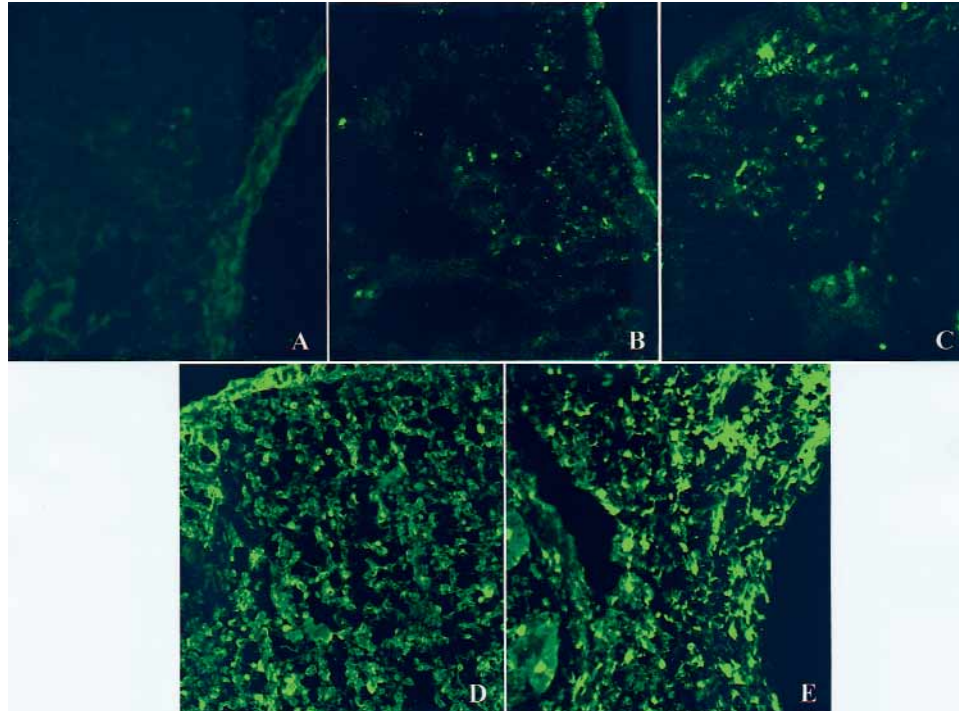


FIG 2. Time-dependent increase in the number of BrdU-positive cells in the presence of 0.1 $\mu\text{g/mL}$ LPS before addition of BrdU (**A**) or 2 (**B**), 6 (**C**), 12 (**D**), or 24 (**E**) hours after incubation with BrdU (10 $\mu\text{mol/L}$). At 12 and 24 hours after incubation with BrdU, the intensity of BrdU immunoreactivity was 40-fold greater when compared with BrdU immunoreactivity at baseline.

$P < .01$), but not CD8⁺ T cells. In addition, 0.1 $\mu\text{g/mL}$ LPS caused an increase in the number of IL-10-positive cells from 5.75 ± 0.69 positive cells per field to 38.16 ± 5.48 positive cells per field ($n = 5$, $P < .05$).

LPS had no significant effect on MBP, IL-4, IL-5, IL-13, or TLR4 immunoreactivity. LPS (1 $\mu\text{g/mL}$) caused significant inflammation and an increase in cytokine immunoreactivity above baseline values ($n = 6$, $P < .001$); however, at this dose, epithelial shedding and tissue distortion was observed (Fig 1, C). For this reason, 0.1 $\mu\text{g/mL}$ LPS was chosen as the optimal concentration for LPS-induced cellular inflammation, and this dose of LPS was used in the remainder of studies. Stimulation of nonatopic tissue with allergen (ragweed, 1000 protein nitrogen units, Hollister-Stier) had no effect on cytokine immunoreactivity or TLR4 expression 24 hours after stimulation (results not shown).

Effect of LPS on IL-2, IL-12, and IFN- γ mRNA expression

Basal expression of IL-2 mRNA, IL-12 mRNA, and IFN- γ mRNA was detected in unstimulated tissue. Incubation of tissue with 0.1 $\mu\text{g/mL}$ LPS caused a significant increase in the number of IL-2 mRNA-positive cells from 9.60 ± 3.04 positive cells per field in unstimulated tissue to 35.90 ± 4.10 positive cells per field ($n = 10$, $P < .001$). LPS (0.1 $\mu\text{g/mL}$) also caused an increase in the number of IFN- γ mRNA-positive cells from 3.30 ± 1.07 to 26.30 ± 4.98 cells per field ($n = 10$, $P < .001$) and an

increase in the number of IL-12 mRNA-positive cells from 2.40 ± 0.79 to 32.50 ± 4.67 positive cells per field 24 hours after incubation ($n = 10$, $P < .001$).

Effect of LPS on proliferation of T lymphocytes

LPS (0.1 $\mu\text{g/mL}$) stimulated an increase in the number of BrdU-positive cells (Fig 2). This proliferation was seen as early as 2 hours after ex vivo incubation with LPS (Fig 2, B) when compared with unstimulated tissue (Fig 2, A). The proliferation was increased in a time-dependent fashion, reaching maximal proliferation at 12 hours (Fig 2, D), and was maintained at 24 hours (Fig 2, E). At 6 hours, the intensity of BrdU immunostaining was increased by greater than 5-fold when compared with the BrdU intensity at time 0. At 12 and 24 hours after incubation with BrdU, this intensity was increased by 40-fold.

By using double immunofluorescence, the number of cells positive for both BrdU and CD3 immunostaining increased progressively with time. At 2 hours, only approximately 10% of the total BrdU-positive cells were RPE-positive CD3⁺ T cells (Fig 3, C), whereas at 24 hours, greater than 70% of the total BrdU-positive cells were CD3⁺ T cells (Fig 3, F). The intensity of BrdU staining at 24 hours after incubation with LPS (Fig 3, D) was greater than 40-fold more intense than the BrdU immunostaining at 2 hours after LPS (Fig 3, A). The intensity of RPE-positive CD3⁺ immunostaining remained constant throughout the time course studied (Fig 3, B and E).

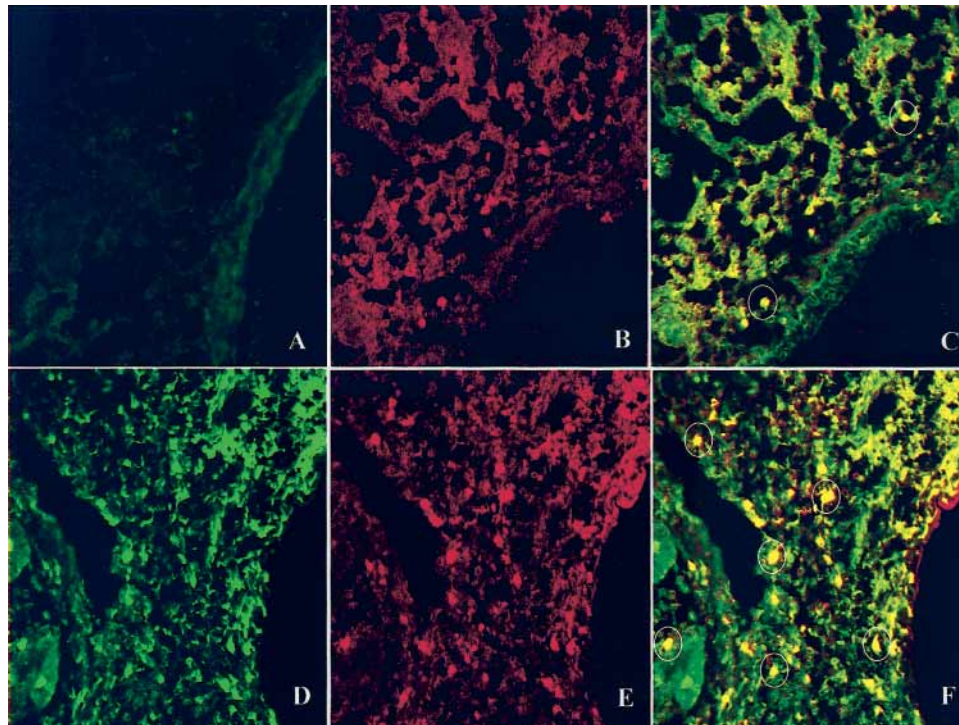


FIG 3. Detection of BrdU-positive proliferating cells (A and D; green), CD3⁺ T cells (B and E; red), and colocalized BrdU-positive/CD3⁺ cells (C and F; yellow) at 2 (top) and 24 (bottom) hours after addition of BrdU in nasal mucosa pretreated with LPS (0.1 μ g/mL). Fig 3, C, indicates 10% colocalization, and Fig 3, F indicates 70% colocalization.

DISCUSSION

Although the ability of bacterial products to act as immunomodulators has previously been reported by us in animals^{2,8} and by others in human subjects,^{9,10} the precise molecular mechanisms that might be responsible for its effects are currently unknown. In particular, the inflammatory cells, cytokines, and receptors that might play an important role in LPS-induced immunomodulation in children remain to be determined. In addition, whether these effects are mediated by systemic and non-specific switch to T_H1 cytokine production or whether these events are mediated locally has not been previously studied.

We found that even in absence of de novo cellular recruitment, LPS caused a shift toward a T_H1 phenotype in the nasal mucosa of children 24 hours after exposure. This change was associated with a dose-dependent increase in macrophage, neutrophil, T-cell, and mast cell numbers and a dose-dependent increase in the number of cells expressing IL-2, IL-12, and IFN- γ (Fig 1). In addition, LPS upregulated the mRNA expression of all of these T_H1 cytokines without altering the expression of the T_H2 cytokines IL-4, IL-5, and IL-13. These data clearly suggest that LPS is working locally to stimulate the development of a T_H1-skewed environment, and this modulation of the immune response is not dependent on cellular recruitment from the systemic circulation.

Local administration of LPS led to a marked increase

in the number of CD3⁺ T cells. After only 2 hours of incubation, these T cells showed evidence of activation, as indicated by increased CD25 immunoreactivity, and rapidly proliferated, as demonstrated by increased BrdU immunodetection. T-cell proliferation was maximal 12 hours after LPS stimulation but continued for at least 24 hours (Fig 2). Double-immunofluorescence studies revealed that CD3⁺ T cells accounted for more than 70% of the total proliferating cells at 24 hours (Fig 3). To our knowledge, LPS-induced T-cell proliferation in young nonatopic children has not been previously reported. The remarkable proliferation of these cells raises questions as to their function. It seems likely that these cells are CD4⁺CD25⁺ T cells,¹¹ naturally occurring immunoregulatory T cells previously shown to inhibit intestinal inflammation in an animal model of colitis.¹² Consistent with this notion, we found increases in the number of CD25⁺ and CD4⁺ T cells but not the number of CD8⁺ T cells after LPS. In addition, we observed a substantial increase in IL-10–positive cells, and CD4⁺CD25⁺ T cells are known to be a good source of IL-10¹³; however, definitive demonstration will require additional experiments.

Another important potential source of IL-10 in this system might be the mast cell, which was also increased in numbers after LPS administration, as demonstrated by means of tryptase staining. Of note, distinctive extracellular tryptase staining was observed around mast cells, suggesting they are actively releasing their granular content in the explants. In this regard our observation that in the nasal

mucosa there is a constitutive expression of TLR4 and that its immunoreactivity is increased after LPS stimulation might provide a possible mechanism by which mast cells are becoming activated. TLR4 is the primary receptor for activation of LPS in a wide variety of cells and plays an important role in innate recognition of bacteria. It has previously been reported that mast cells express the TLR4 receptor, and activation of TLR4 is essential for mast cell-mediated innate immune responses.¹⁴ Moreover, we and others have previously shown that LPS is capable of releasing prestored IL-10 from mast cells.^{8,15}

Although beyond the scope of this study, it is possible that the marked proliferative responses to local LPS application occurred because we used explants from young children. The possibility that the local immune response is more plastic in children than in adults will require additional experiments aimed at comparing explants from different age groups. However, there is good reason to believe that this might be the case, as shown by our data and also supported by epidemiology studies that suggest that reduced exposure of children to microbial stimuli might be responsible for their dramatic increase in the incidence of asthma.^{16,17} This hygiene hypothesis has been documented only in young children and not in adults.

In summary, local administration of LPS to explanted nasal mucosa from nonatopic children leads to a shift in the immune environment to a T_H1 phenotype by activating inflammatory cells and promoting proliferation of T cells in the absence of systemic recruitment of inflammatory cells. These findings suggest that the local action of bacterial products on the mucosa surface is enough to change the phenotype of the immune response and underscore the importance of LPS in tissue-specific immunity in early life. Our results show that this explant model exhibits many of the features of LPS-induced *in vivo* inflammation previously described in both animals and human subjects, including inflammation, cytokine activation, cellular proliferation, and mast cell degranulation. This model will form a basis for ongoing studies in which nasal mucosa biopsy specimens from atopic children will be used to closely examine the mechanisms involved in bacterial-induced modification of allergic inflammation during the early sensitization process in young children.

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REFERENCES

- Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002;109:379-92.
- Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge following exposure to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 2000;22:604-12.
- Cameron L, Christodoulou P, Lavigne F, Nakamura Y, Eidelman D, McEuen A, et al. Evidence for local eosinophil differentiation within allergic nasal mucosa: inhibition with soluble IL-5 receptor. *J Immunol* 2000;164:1538-45.
- Cameron L, Hamid Q, Wright E, Nakamura Y, Christodoulou P, Muro S, et al. Local synthesis of ϵ germline gene transcripts, IL-4, and IL-13 in allergic nasal mucosa after *ex vivo* allergen exposure. *J Allergy Clin Immunol* 2000;106:46-52.
- Frew AJ, Kay AB. The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988;141:4158-64.
- Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, et al. Expression of mRNA for interleukin-5 in mucosa bronchial biopsies from asthma. *J Clin Invest* 1991;87:1541-6.
- Hamid Q, Wharton J, Terenghi G, Hassall CJ, Aimi J, Taylor KM, et al. Localization of atrial natriuretic peptide mRNA and immunoreactivity in the rat heart and human atrial appendage. *Proc Natl Acad Sci U S A* 1987;84:6760-4.
- Tulic MK, Knight DA, Holt PG, Sly PD. Lipopolysaccharide inhibits the late-phase response to allergen by altering nitric oxide synthase activity and interleukin-10. *Am J Respir Cell Mol Biol* 2001;24:640-6.
- Gereda JE, Leung DYM, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 2000;355:1680-3.
- von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Malsch S, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000;30:1230-4.
- Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 1998;188:287-96.
- Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995-1003.
- Annacker O, Pimenta-Araujo R, Buren-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol* 2001;166:3008-18.
- Supajatura V, Ushio H, Nakao A, Okumura K, Ra C, Ogawa H. Protective roles of mast cells against anterobacterial infection are mediated by toll-like receptor 4. *J Immunol* 2001;167:2250-6.
- Malaviya R, Georges A. Regulation of mast cell-mediated innate immunity during early response to bacterial infection. *Clin Rev Allergy Immunol* 2002;22:189-204.
- von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Roell G, Thiemann HH. Prevalence of asthma and atopy in two areas of West and East Germany. *Am J Respir Crit Care Med* 1994;149:365-70.
- Martinez FD. Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? *Thorax* 1994;49:1189-91.