

Bacterial DNA in house and farm barn dust

Sitesh R. Roy, MD, Allison M. Schiltz, BA, Alex Marotta, MD, Yiqin Shen, BA, and Andrew H. Liu, MD *Denver, Colo*

Background: Early in life, natural exposure to microbial components (eg, endotoxin) may mitigate allergy and asthma development in childhood. Bacterial DNA is a potent stimulus for the innate immune system; its immune stimulatory potential in dust is unknown.

Objectives: We sought to quantify bacterial DNA and endotoxin in content in dust from urban homes, rural homes, farm homes, and farm barns and to determine if dust DNA is immune-stimulatory.

Methods: Total DNA, bacterial DNA, and endotoxin were measured in 32 dust samples. To measure bacterial DNA content, a quantitative polymerase chain reaction assay specific for bacterial ribosomal DNA was developed. Peripheral blood mononuclear cells from 5 adults were stimulated with endotoxin-free dust DNA with/without lipopolysaccharide (LPS) from selected dust samples. IL-12p40, IL-10, and tumor necrosis factor- α were measured in cell supernatants by enzyme-linked immunosorbent assay.

Results: Bacterial DNA in dust correlated with endotoxin ($r = 0.56$, $P < .001$) and total DNA content ($r = 0.51$, $P = .003$). The highest bacterial DNA levels were measured in farm barns (mean, 22.1 $\mu\text{g/g}$ dust; range, 1.3 to 56.2), followed by rural homes (6.3 $\mu\text{g/g}$; 0.2 to 20), farm homes (2.2 $\mu\text{g/g}$; 0.1 to 9.1), and urban homes (0.6 $\mu\text{g/g}$; 0.1 to 1.2). Farm barn DNA significantly potentiated ($P \leq .05$) LPS-induced IL-10 and IL-12 p40 but not tumor necrosis factor- α release (13-fold, 3-fold, and 1.5-fold increases, respectively). DNA from 6 urban homes did not demonstrate this LPS-potentiating effect.

Conclusions: Endotoxin is a marker for bacterial DNA, which is also higher in locales of lower asthma and allergy prevalence. DNA from farm barn dust augments the immune modulatory effects of endotoxin and may combine with exposure to other such naturally occurring microbial components to mitigate allergy and asthma development. (*J Allergy Clin Immunol* 2003;112:571-8.)

Key words: Farm barn, dust, DNA, bacterial DNA, endotoxin, IL-10, IL-12, hygiene, CpG DNA

Abbreviations used

CpG:	Cytosine-phosphate-guanine
EU:	Endotoxin unit
IL-12 p40:	Interleukin 12, p40 subunit
LAL:	Limulus amoebocyte lysate (assay)
LPS:	Lipopolysaccharide
ODN:	Oligodeoxynucleotide
PBMC:	Peripheral blood mononuclear cells
qRT PCR:	Quantitative real-time polymerase chain reaction
TLR:	Toll-like receptor

Natural exposure to microbial components early in life might protect against the development of atopy and asthma. Such exposure also plays an important role in occupational lung diseases and can augment established allergic and asthmatic inflammation. Endotoxin, a cell wall component of gram-negative bacteria, has been a prototypical microbial component in this role.^{1,2} House dust endotoxin levels have been found to correlate with protective T_H1 -type immune development and lack of allergen sensitization in a cohort of early wheezers.³ A closer look at rural and farming communities, where atopy and asthma are less prevalent, has reinforced this association between higher house dust endotoxin levels and lower risk of atopic diseases, including atopy-associated asthma.⁴⁻⁶ We wondered if other hardy microbial components might contribute to the immune stimulatory capacity of house and farm barn dust.

Bacterial DNA is an excellent candidate because of its hardy nature and immune-stimulatory properties. It contains many unmethylated cytosine-phosphate-guanine (CpG) motifs, which directly stimulate innate immune cells through Toll-like receptor 9 (TLR9).⁷⁻⁹ Bacterial DNA and synthesized CpG oligodeoxynucleotides (CpG ODN) that mimic bacterial DNA CpG motifs are strong inducers of T_H1 -type immune responses.¹⁰⁻¹³ CpG ODN can inhibit allergen-induced (T_H2) airway inflammation in murine asthma models.¹⁴⁻¹⁶ CpG ODN coadministered with an antigen induces a strong T_H1 -type immune response or a switch from T_H2 to T_H1 -type immune response to that antigen.¹⁷⁻²⁰ The routine use of DNA assays in forensics and ecological microbiology is evidence of DNA durability and persistence in the environment. Thus, we sought to quantify bacterial DNA in dust from various locales and to evaluate its immune-stimulatory potential.

From the Division of Pediatric Allergy and Immunology, National Jewish Medical and Research Center, and the Department of Pediatrics, University of Colorado Health Sciences Center, Denver.

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Reprint requests: Andrew H. Liu, MD, Pediatric Allergy and Immunology, National Jewish Medical and Research Center, 1400 Jackson St (K1023), Denver, CO 80206.

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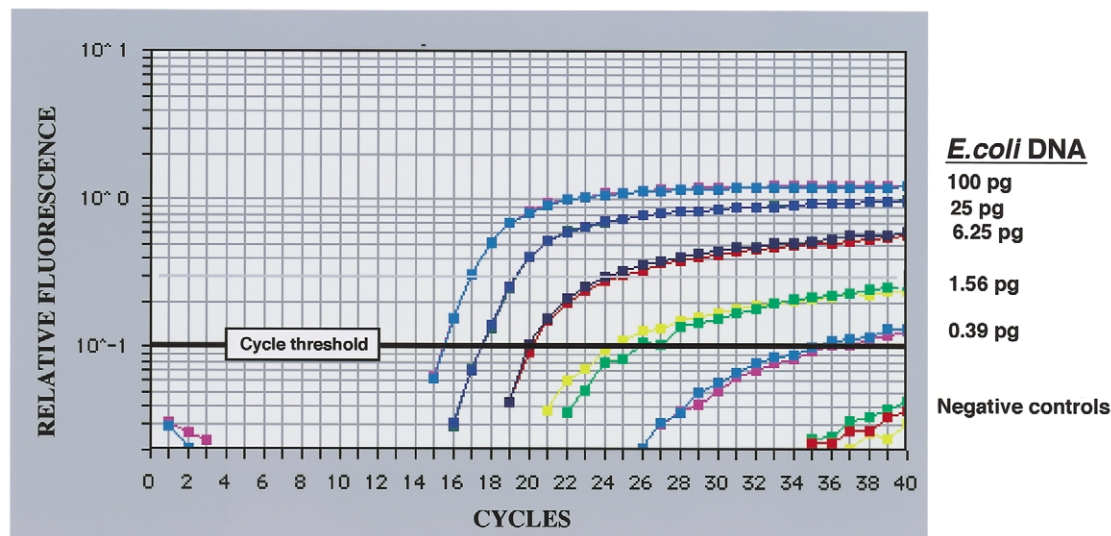


FIG 1. Amplification of conserved bacterial segment in ribosomal operons of *E coli* DNA by qRT PCR. This exemplifies how standard curves were generated in each PCR run to quantify bacterial DNA in dust samples. Duplicate samples of 4-fold serial dilutions of *E coli* genomic DNA were used as templates for a 2-step nested PCR. Starting amount of *E coli* DNA is shown next to each amplification curve. Relative fluorescence was measured; cycle threshold at which threshold fluorescence was reached is shown.

METHODS

DNA extraction and purification from dust samples for quantification and cell stimulation

Thirty-two dust samples from 4 different locales (ie, farm barns, farm homes in the United States, rural homes in India, and urban homes in Denver) were collected. DNA was extracted from these dust samples by using the UltraClean Soil DNA Kit (MoBio Laboratories Inc, Solana Beach, Calif), with slight modifications to maximize the DNA yield. DNA was further purified through the use of the Qiagen Plasmid Midi Kit (QIAGEN Inc, Valencia, Calif) to remove proteins and humic acid that coextract with dust DNA and can interfere with DNA polymerase enzyme activity. The extracted DNA was suspended in 100 μ L of Tris-EDTA buffer.

The total DNA content of each sample was estimated by UV spectrophotometry (DU-65 Spectrophotometer, Beckman, Fullerton, Calif). Samples were read at the following wavelengths: 230 λ for humic acid contamination; 260 λ for DNA content; and 280 λ for protein contamination. The mean 260/280 ratio was 1.65 ± 0.07 (range, 1.45 to 1.99), and the 260/230 ratio was 1.61 ± 0.08 (range, 1.1 to 2.0). These ratios indicated low levels of humic acid and protein contamination, considered to be suitable for PCR.²¹

For 2 farm barns and 6 urban home dust samples, DNA was extracted from 5 to 8 grams of dust in batches and purified with the QIAGEN Endofree Plasmid Maxi Kit (QIAGEN Inc, Valencia, Calif) to optimize endotoxin removal. Pure DNA was obtained after 1 or 2 runs through the endotoxin removal kit. The mean endotoxin content of this DNA, as measured by the limulus amoebocyte lysate (LAL) assay, ranged from 0.03 to 0.006 endotoxin unit (EU)/ μ g DNA for the various batches. By UV spectrophotometry, the 260/280 ratio was >1.8 and the 260/230 ratio was >2 for all of the batches of DNA extracted, indicating high DNA purity without protein or humic acid contamination.^{22,23} This endotoxin-free DNA was used for in vitro peripheral blood mononuclear cell (PBMC) stimulations.

Quantification of bacterial DNA in dust samples

A 2-step, nested PCR quantified bacterial DNA in dust samples with primers and probes specific for conserved bacterial sequences in the 16S and 23S ribosomal operons.

The first nested PCR amplification targeted conserved bacterial sequences in the 16S and 23S genes with the universal bacterial primers 785 (forward) and 422 (reverse).²⁴ This initial PCR was performed on 2 μ L of DNA extracted from dust in 50- μ L reactions containing AmpliTaq low-DNA 1.25 U (Applied Biosystems, Foster City, Calif), 1 $\times$ GeneAmp PCR Buffer II (Applied Biosystems), dNTP 0.2 mmol/L each, MgCl₂ 3 mmol/L, and primers 785 and 422 0.015 μ g each (Synthegen, Houston, Tex). Amplification consisted of 15 cycles of 94°C for 1 minute, 42°C for 2 minutes, and 72°C for 3 minutes in a Gene Amp 9600 PCR System (Perkin Elmer, Boston, Mass).²⁴ Reaction products, analyzed by 2% agarose gel electrophoresis, were visualized as a single band of ~1200 to 1500 base pairs.

The second amplification was by quantitative real-time PCR (qRT PCR) with primers (785 and 1512r)²⁵ and probe (1400r)²⁶ targeting conserved bacterial sequences in the 16S gene. Each 50 μ L reaction contained 2 μ L of PCR product from the first amplification, AmpliTaq low-DNA 1.25 U, 1 $\times$ GeneAmp PCR Buffer II, dNTP 0.2 mmol/L each, MgCl₂ 5 mmol/L, primers 785 and 1512r 0.8 μ mol/L each (Synthegen), Rox Standard II 0.06 μ mol/L (Synthegen), and 1400r probe 0.1 μ mol/L (Synthegen). The qRT PCR was run on an ABI Prism 7700 PCR system (Applied Biosystems, Foster City, Calif) with an initial cycle of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 52°C for 1 minute, and 72°C for 2 minutes. The relative fluorescence, which is the increase in reporter dye intensity relative to the passive internal reference dye, was measured. The PCR data were analyzed by means of the ABI Sequence Detection System software from Applied Biosystems. As expected, reaction products yielded a single band of ~700 to 800 base pairs on 2% agarose gel electrophoresis (data not shown).

Serial dilutions of *Escherichia coli* DNA, extracted from lyophilized cells of strain B *E coli* (Sigma, St Louis, Mo), were used

to generate a standard curve for each qRT PCR run (Fig 1). Standard curves, generated by correlating the known input amount of *E coli* DNA with its cycle threshold (coefficient of determination, or $R^2 \geq 0.95$), were used to convert the cycle threshold for each dust DNA extract to the amount of bacterial DNA in picograms. Each dust DNA extract was run in duplicate and at 2 dilutions in the detection range defined by the standard curves. For various dust samples, the intra-assay R^2 was 0.91 ($n = 23$; $P < .0001$) and the interassay R^2 was 0.96 ($n = 9$; $P < .0001$).

Measurement of endotoxin levels in dust samples

Endotoxin concentrations of the dust and DNA samples were measured by means of an LAL assay (Bio-Whittaker QCL-1000, Walkersville, Md) under pyrogen-free conditions, as previously described.³ Endotoxin concentrations are expressed in endotoxin units per milligram of dust.

In vitro human PBMC stimulations with DNA

PBMC were isolated from the peripheral blood of healthy volunteers by density centrifugation with Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) in Accuspin tubes (Sigma Diagnostics Inc, St Louis, Mo). The cells were suspended in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 1.5 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Grand Island, NY). All reagents were selected for lowest endotoxin content; 24-hour cell cultures were performed in 96-well round-bottom plates (200 µL per well) at a final concentration of 1×10^6 cells per mL in a 5% CO₂ humidified incubator at 37°C. Five wells were run for each stimulation condition. Cell supernatants were removed and stored at -20°C. This study was approved by the Institutional Review Board of the National Jewish Medical and Research Center, Denver, Colo.

ELISA assays for IL-12 p40, IL-10, and TNF-α

Human IL-12 p40, IL-10, and TNF-α ELISA (OptEIA PharMin-gen, San Diego, Calif) were performed in Immulon 4-HBX plates (Dynex Technologies, Chantilly, Va). All supernatant samples were run in duplicate. The lower limit of detection was 15 pg/mL for IL-12 p40 and 8 pg/mL for IL-10 and TNF-α.

Statistical analyses

For comparisons with small subject numbers between groups (ie, $n \leq 20$) and/or nonnormal distribution of data, the nonparametric Wilcoxon rank sum test was used. For correlation analyses, the data were log-transformed to normalize its distribution, and pairwise correlations (r = Pearson coefficient) were obtained. Significance was set at a P value of .05.

RESULTS

DNA was extracted from 32 dust samples from 4 locales: farm barns and farm homes in the United States, rural homes in India, and urban homes in Denver. The farm settings included a turkey farm, a dairy farm, and some ranches. The farm barns were residences for different types of livestock, including cows, horses, goats, fowl, cats, dogs, and rodents. Most farm homes had multiple furry pets. The India homes were in rural villages. Almost all had furry pets, birds (chickens, ducks), and some livestock (cows, goats) in the home and also outside of the home. Of the Denver homes, most had a pet (usually a dog and/or a cat).

Total DNA in dust

Total DNA concentrations, estimated spectrophotometrically, varied widely and did not differ significantly by locale ($P = .56$). Farm barns had a mean of 46.5 µg total DNA/g dust (SD, 38.9; range, 5.4 to 106.6), rural homes had 31.1 µg/g dust (SD, 31.8; range, 6.0 to 104.2), urban homes had 18.2 µg/g dust (SD, 10.3; range, 6.3 to 37.4), and farm homes had 16.6 µg/g dust (SD, 6.4; range, 9.4 to 25.3).

Bacterial DNA in dust

The bacterial DNA content of dust samples was measured by using a quantitative PCR assay with primers specific for bacterial ribosomal DNA.²⁷ Serial dilutions of *E coli* DNA yielded the standard curve. All dust samples contained detectable levels of bacterial DNA, which differed significantly by locale ($P = .001$; Fig 2). The highest levels of bacterial DNA were in the farm barns, with a mean of 22.1 µg/g barn dust (SD, 21.8; range, 1.3 to 56.2), followed by rural homes with 6.3 µg/g dust (SD, 6.9; range, 0.2 to 20), farm homes with 2.2 µg/g dust (SD, 3.2; range, 0.1 to 9.1), and urban homes with 0.6 µg/g dust (SD, 0.4; range, 0.1 to 1.2). The bacterial DNA content positively correlated with the total DNA content ($r = 0.51$; $P = .003$). By dividing the bacterial DNA content (quantified by qRT PCR) by the total DNA (quantified by spectrophotometry), the proportion (%) of bacterial DNA was calculated. Bacterial DNA percentage by locale (highest to lowest) is as follows: mean of 71% (range, 10% to 208%) for farm barns, 31% (1% to 80%) for rural homes, 10% (1% to 36%) for farm homes, and 3% (1% to 8%) for urban homes ($P = .007$).

Endotoxin in dust

Endotoxin was detectable in all dust samples. Endotoxin concentration differed significantly by locale ($P < .001$; Fig 2), consistent with previous results.⁴ The farm barns had the highest endotoxin levels, with a mean of 2145 EU/mg dust (SD, 1902; range, 400 to 4907), followed by farm homes with 489 EU/mg dust (SD, 390; range, 75 to 1250), rural homes with 345 EU/mg dust (SD, 234; range, 71 to 712), and urban homes with 108 EU/mg dust (SD, 46; range, 45 to 200). The bacterial DNA content of the dust samples positively correlated with the endotoxin levels ($r = 0.56$, $P < .001$; Fig 3). Total DNA in dust samples did not correlate significantly with endotoxin levels ($r = 0.33$, $P = .06$).

PBMC stimulation with dust DNA with/without lipopolysaccharide

Endotoxin-free dust DNA samples from 2 farm barns and 6 urban homes were used to stimulate PBMC from 5 volunteers. The DNA samples from both locales were non-stimulatory for IL-12 p40, IL-10, and TNF-α release at the 3-µg/mL dose (Fig 4). These cytokine levels were comparable to a synthetic phosphorothioated CpG ODN (ODN 2006) optimized for stimulation of human PBMC.^{28,29} At the 10-µg/mL dose, barn DNA tended to be slightly stim-

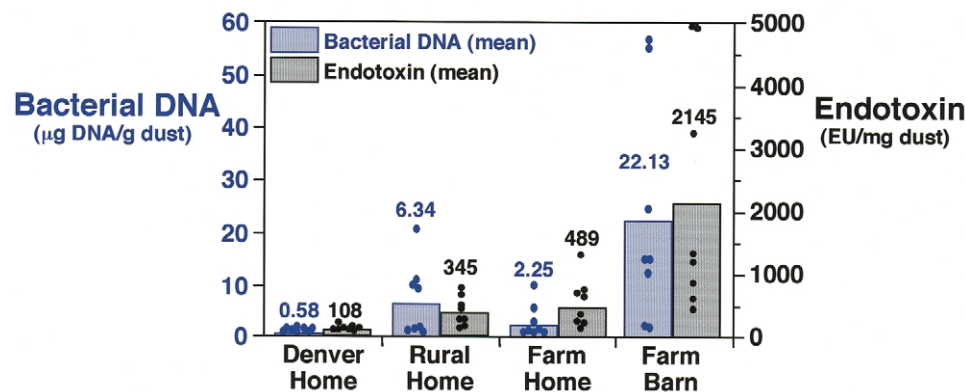


FIG 2. Bacterial DNA and endotoxin content in dust samples from 4 different locales: (1) urban homes in Denver, Colo (n = 8); (2) rural homes in India (n = 8); (3) farm homes in the United States (n = 8); and (4) US farm barns (n = 8). Bacterial DNA (*left side, blue*) was measured with qRT PCR assay. Endotoxin (*right side, black*) was measured with LAL assay. Bacterial DNA and endotoxin levels in dust samples differed significantly by locale, with highest levels in farm barn samples and lowest levels in urban homes ($P \leq .001$).

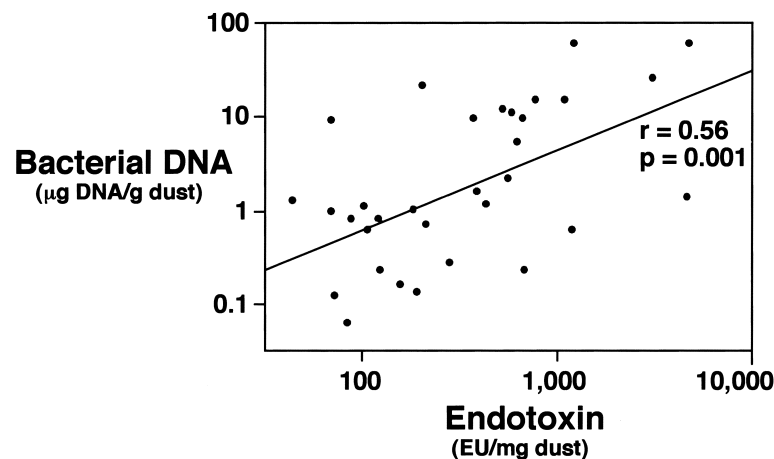


FIG 3. Correlation of bacterial DNA and endotoxin in dust samples. Log-transformed levels of bacterial DNA and endotoxin were positively correlated (pairwise correlation).

ulatory for IL-12 and TNF- α release compared with CpG ODN, which was less stimulatory, and urban DNA, which remained nonstimulatory (data not shown). Barn and urban DNA alone at the higher dose of 10 μ g/mL did not stimulate IL-10 release (data not shown).

To assess the potential of barn and home DNA to augment lipopolysaccharide (LPS)-induced cytokine release, PBMC were stimulated with DNA and small amounts of LPS. Costimulations with 3 μ g/mL of urban home DNA and 300 pg/mL LPS elicited no significant increase in IL-12 p40, IL-10, or TNF- α release over that induced by 300 pg/mL of LPS alone (Fig 4). Combining 3 μ g/mL of barn DNA with 300 pg/mL of LPS led to a 13-fold increase in IL-10 release ($P = .03$), a 3-fold increase in IL-12 p40 release ($P = .05$), and a 1.5-fold increase in TNF- α release ($P =$ not significant, NS) over the 300-pg/mL LPS and the 3- μ g/mL urban home DNA + 300 pg/mL LPS stimulations (Fig 4). Combining 10 μ g/mL of the optimized CpG ODN with 300 pg/mL LPS also stimulated a 5-fold increase in IL-10 and a 3-fold

increase in IL-12p40 release over LPS alone and urban DNA + LPS. The combination of 10 μ g/mL of barn DNA and 300 pg/mL of LPS induced IL-12 and IL-10 release that approached maximal LPS-induced (10 ng/mL) stimulation of these cytokines (data not shown).

DISCUSSION

This is the first paper to describe the isolation of DNA from house and farm barn dust, the quantification of its bacterial DNA content, and the assessment of its immune-stimulatory capacity. There were widely variable amounts of total DNA in dust samples from different locales, ranging from 5 to 106 μ g/g dust. Although no published study to our knowledge has reported the DNA content of house or farm barn dust, several studies that used similar extraction methods have quantified total DNA content of soil, with a range from 15 to 546 μ g/g of soil.^{23,30} Bacterial DNA content in dust samples from different locales was also widely variable, ranging from

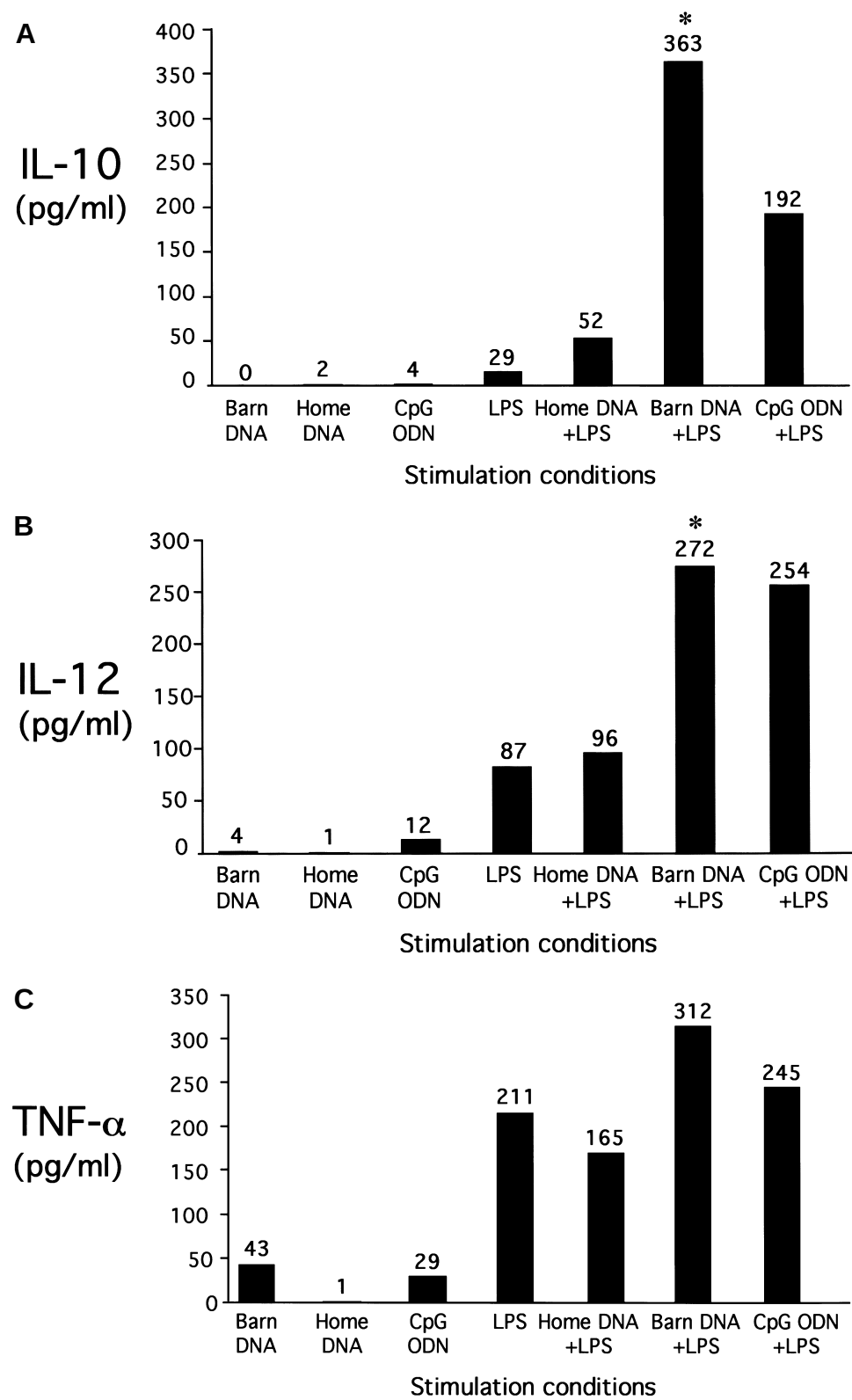


FIG 4. Cytokine release from in vitro stimulations of PBMC from 5 human subjects. Mean IL-10 (**A**), IL-12p40 (**B**), and TNF- α (**C**) release stimulated by DNA (3 μ g/mL) from dust of 2 farm barns and 6 urban homes, with and without 300 pg/mL LPS, is shown; 300 pg/mL LPS alone and 10 μ g/mL CpG ODN 2006^{28,29} with and without 300 pg/mL LPS are included as controls. Farm barn DNA showed significant potentiation of IL-10 (13-fold) and IL-12 (3-fold) release when combined with low levels of LPS, above that stimulated by LPS alone and urban home DNA plus LPS (* $P \leq .05$, Wilcoxon).

0.1 to 56 $\mu\text{g/g}$ dust. Endotoxin levels in dust, which also varied by locale, correlated positively with bacterial DNA content. Hence, these bacterial byproducts might be markers of microbial load in the environments from where the dust samples were collected.

When comparing the total DNA, bacterial DNA, and endotoxin content of dust samples by their locales, significant differences in both bacterial DNA and endotoxin levels were observed. The highest levels were generally found in farm barns; the lowest levels were in urban homes. The number of dust samples in this study were too few to glean conclusions on environmental factors contributing to higher or lower bacterial DNA levels in dust. Previous studies have found that the endotoxin content in house dust was associated with cats, dogs, and other animals in the home.³¹⁻³⁴ For farm homes, dust endotoxin levels correlated with their associated farm barn dust endotoxin levels, suggesting that significant amounts of dust endotoxin in farm homes comes from their barns.⁴

Coexistence of high levels of bacterial DNA and endotoxin in some dust samples, especially from farm barns and rural homes, raises intriguing possibilities of synergy with regard to immune modulatory, atopy-protective, and/or pro-inflammatory effects. Bacterial CpG DNA and endotoxin are both pathogen-associated molecular patterns and ligands for TLR9 and TLR4, respectively. Both induce the activation of nuclear factor- κB and multiple mitogen-associated protein kinases through a shared MyD88-dependent pathway.^{9,35} TLR9 and TLR4 are both expressed by immune cells, including human monocytes, dendritic cells, and B-lymphocytes, although the amount of these TLRs expressed on different immune cell types varies.³⁶⁻³⁸ There are other interesting differences as well. For example, endotoxin can also induce cell activation through an MyD88-independent pathway; current evidence has not revealed an alternative activation pathway for CpG DNA.^{9,35} TLR4 is expressed on cell surfaces and interacts with other enhancer proteins that bind endotoxin (CD14, LPS-binding protein, MD-2).³⁵ In comparison, current evidence has localized TLR9 to endosomal compartments, suggesting that TLR9 is primarily an intracellular receptor in normal conditions.⁹

Endotoxin-free dust DNA alone did not stimulate human PBMC at 3 $\mu\text{g/mL}$ and was only slightly immunostimulatory at higher doses (10 $\mu\text{g/mL}$). This is consistent with previous studies of human PBMC stimulated with endotoxin-free bacterial DNA and/or CpG ODN that have reported either low levels of TNF- α release³⁹ or were unable to detect TNF- α release.²⁸ Hence, more sensitive measures of intracellular cytokine synthesis upregulation, such as PCR (for cytokine mRNA) and flow cytometry (to detect intracellular cytokine staining), have been used to detect IL-12, IL-18, IFN- γ , and TNF- α expression in response to CpG DNA.^{28,40}

We have demonstrated potentiation of especially IL-10 and also IL-12 p40 release from PBMC by costimulation with barn dust DNA and LPS. This enhancement of LPS-induced cytokine release by using bacterial DNA or CpG

ODNs has been reported for IL-12, IFN- γ , TNF- α , and nitric oxide in murine studies.⁴¹⁻⁴⁴ In human PBMC, synergy has been shown for in vitro IL-6 release by using CpG ODN with LPS,²⁸ but similar published information for IL-12, IL-10, and TNF- α release is lacking, to our knowledge. The marked increase in IL-10 production stimulated by farm barn DNA plus LPS could play a major role in the regulation of overall immune stimulation seen with this combination. IL-10 is an important regulatory cytokine that has the capacity to minimize LPS-induced inflammation and T_H2-mediated inflammatory processes.⁴⁵⁻⁴⁷ Selective induction of IL-12 but not TNF- α by certain CpG ODNs has been reported.⁴⁸ IL-10 and IL-12 release potentiated by farm barn DNA and endotoxin could prime the acquired immune system for T-regulatory and modified T_H1-type immune responses to other proteins and foreign antigens that it encounters, thus preventing T_H2-mediated atopy and asthma.^{1,49,50}

Urban home DNA, when combined with LPS, did not increase IL-10, IL-12 p40, or TNF- α release. One possible explanation for this might be the low bacterial DNA content. Very low proportions of total DNA from urban homes were bacterial in origin (~3%). These low levels of bacterial DNA might not be sufficient to demonstrate a synergistic effect when combined with LPS. Additionally, the nonbacterial DNA in dust samples may alter the immune-stimulatory capacity of dust. Most eukaryotic and especially vertebrate DNA has been shown to contain 20-fold fewer CpG motifs than bacterial DNA, of which the majority are methylated.^{51,52} Such eukaryotic DNA does not possess immune stimulatory potential and might even be immune inhibitory.^{53,54} Mammalian DNA also contains neutralizing motifs (CpG-N) that antagonize the effects of immune-stimulatory CpG motifs.^{55,56}

There is already tremendous interest in the immunomodulatory potential of CpG motifs found in bacterial DNA for the treatment of cancer and infectious diseases, development of enhanced vaccines, and prevention or cure of allergic diseases and asthma. Therapeutic applications are being explored in animal studies and human trials involving allergen immunotherapy to short ragweed allergen *Amb a1*, treatment of allergic rhinitis and asthma, enhanced infectious disease vaccines, and cancer immunotherapy.^{9,57-60} With the understanding that allergic diseases and asthma have their origins early in life, the study of immune-stimulatory DNA in dust is another step toward clarifying how natural exposure to microbial components influences early immune development.

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REFERENCES

1. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 2002;109:379-92.
2. Reed CE, Milton DK. Endotoxin-stimulated innate immunity: a contributing factor for asthma. *J Allergy Clin Immunol* 2001;108:157-66.
3. 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 sensitization in infants at high risk of asthma. *Lancet* 2000;355:1680-3.
4. Gereda JE, Leung DYM, Liu AH. House-dust endotoxin is higher in rural homes in developing countries and farm homes, where asthma is less prevalent. *JAMA* 2000;284:1652-3.
5. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Malsch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;358:1129-33.
6. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;347:869-77.
7. Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. *J Leukoc Biol* 2002;71:538-44.
8. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;408:740-5.
9. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002;20:709-60.
10. Fujieda S, Iho S, Kimura Y, Yamamoto H, Igawa H, Saito H. Synthetic oligodeoxynucleotides inhibit IgE induction in human lymphocytes. *Am J Respir Crit Care Med* 2000;162:232-9.
11. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 1996;93:2879-83.
12. Krieg AM, Love-Homan L, Yi AK, Harty JT. CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J Immunol* 1998;161:2428-34.
13. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, et al. DNA from bacteria, but not from vertebrates, induced interferons, activates natural killer cells and inhibits tumor growth. *Microbiol Immunol* 1992;36:983-97.
14. Kline JN, Krieg AM, Waldschmidt TJ, Ballas ZK, Jain V, Businga TR. CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 1999;104:1258-64.
15. Kline JN, Waldschmidt TJ, Businga TR, Lemish JE, Weinstock JV, Thorne PS, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998;160:2555-9.
16. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284-93.
17. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (th1) immunity. *J Exp Med* 1997;186:1623-31.
18. Parronchi P, Brugnolo F, Annunziato F, Manuelli C, Sampognaro S, Mavilia C, et al. Phosphorothioate oligodeoxynucleotides promote the in vitro development of human allergen-specific CD4+T cells into Th1 effectors. *J Immunol* 1999;163:5946-53.
19. Roman M, Martin-Orozco E, Goodman JS, Nguyen M, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997;3:849-54.
20. Horner AA, Takabayashi K, Beck L, Sharma B, Zubeldia J, Baird S, et al. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. *J Allergy Clin Immunol* 2002;110:413-20.
21. Stach JEM, Bathe S, Clapp JP, Burns RG. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiol Ecol* 2001;36:139-51.
22. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
23. Yeates C, Gillings MR, Davison AD, Altavilla N, Veal DA. Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online* 1998;1:40-7.
24. Leys EJ, Griffen AL, Strong SJ, Fuerst PA. Detection and strain identification of *Actinobacillus actinomycetemcomitans* by nested PCR. *J Clin Microbiol* 1994;32:1288-94.
25. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S Ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;173:697-703.
26. Fry NK, Fredrickson JK, Fishbain S, Wagner M, Stahl DA. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. *Appl Environ Microbiol* 1997;63:1498-504.
27. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J Clin Microbiol* 2000;38:2362-5.
28. Hartmann G, Krieg AM. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Ther* 1999;6:893-903.
29. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 2000;164:944-53.
30. Burgmann H, Pesaro M, Widmer F, Zeyer J. A strategy for optimizing quality and quantity of DNA extracted from soil. *J Microbiol Methods* 2001;45:7-20.
31. Douwes J, Zuidhof A, Doekes G, van der Zee S, Wouters I, Boezen H, et al. (1'3)- β -D-Glucan and endotoxin in house dust and peak flow variability in children. *Am J Respir Crit Care Med* 2000;162:1348-54.
32. Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, et al. Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. *J Allergy Clin Immunol* 2001;108:847-54.
33. Gereda JE, Klennert MD, Price MR, Leung DYM, Liu AH. Metropolitan home living conditions associated with indoor endotoxin levels. *J Allergy Clin Immunol* 2001;107:790-6.
34. Heinrich J, Gehring U, Douwes J, Koch A, Fahlbusch B, Bischof W, et al. Pets and vermin are associated with high endotoxin levels in house dust. *Clin Exp Allergy* 2001;31:1839-45.
35. Medzhitov R. Toll-like receptors and innate immunity. *Nature Rev Immunol* 2001;1:135-45.
36. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 2002;168:4531-7.
37. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* 2001;194:863-9.
38. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of toll-like receptors in human monocytes and dendritic cells. *J Immunol* 2001;166:249-55.
39. Bohle B, Orel L, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs induce low levels of TNF-alpha in human B lymphocytes: possible adjuvants for Th1 responses. *J Immunol* 2001;166:3743-8.
40. Bohle B, Jahn-Schmid B, Maurer D, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur J Immunol* 1999;29:2344-53.
41. Cowdery JS, Chace JH, Yi AK, Krieg AM. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *J Immunol* 1996;156:4570-5.
42. Gao JJ, Xue Q, Papasian CJ, Morrison DC. Bacterial DNA and lipopolysaccharide induce synergistic production of TNF-alpha through a post-transcriptional mechanism. *J Immunol* 2001;166:6855-60.
43. Gao JJ, Zuvanich EG, Xue Q, Horn DL, Silverstein R, Morrison DC. Cutting edge: bacterial DNA and LPS act in synergy in inducing nitric oxide production in RAW 264.7 macrophages. *J Immunol* 1999;163:4095-9.
44. Yi AK, Yoon JG, Hong SC, Redford TW, Krieg AM. Lipopolysaccharide and CpG DNA synergize for tumor necrosis factor-alpha production through activation of NF-kappaB. *Int Immunol* 2001;13:1391-404.
45. Akdis CA, Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology* 2001;103:131-6.
46. Schwartz DA, Wohlford-Lenane CL, Quinn TJ, Krieg AM. Bacterial DNA or oligonucleotides containing unmethylated CpG motifs can minimize lipopolysaccharide-induced inflammation in the lower respiratory tract through an IL-12-dependent pathway. *J Immunol* 1999;163:224-31.
47. Wakkach A, Cottrez F, Groux H. Can interleukin-10 be used as a true immunoregulatory cytokine? *Eur Cytokine Netw* 2000;11:153-60.
48. Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur J Immunol* 1997;27:3420-6.
49. Akdis M. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;102:98-106.
50. Jeanin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998;160:3555-61.
51. Bird AP. DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res* 1980;8:1499-504.

52. Hergersberg M. Biological aspects of cytosine methylation in eukaryotic cells. *Experientia* 1991;47:1171-85.
53. Chen Y, Lenert P, Weerantna R, McCluskie M, Wu T, Davis HL, et al. Identification of methylated CpG motifs as inhibitors of the immune stimulatory CpG motifs. *Gene Ther* 2001;8:1024-32.
54. Pisetsky DS, Reich CF. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin Immunol* 2000;96:198-204.
55. Han J, Zhu Z, Hsu C, Finley WH. Selection of antisense oligonucleotides on the basis of genomic frequency of the target sequence. *Antisense Res Dev* 1994;4:53-65.
56. Krieg AM, Wu T, Weeratna R, Efler SM, Love-Homan L, Yang L, et al. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc Natl Acad Sci U S A* 1998;95:12631-6.
57. Krieg AM, Davis HL. Enhancing vaccines with immune stimulatory CpG DNA. *Curr Opin Mol Ther* 2001;3:15-24.
58. Tighe H, Takabayashi K, Schwartz D, van Nest G, Tuck S, Eiden JJ, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000;106:124-34.
59. Jain VV, Kitagaki K, Businga T, Hussain I, George C, O'Shaughnessy P, et al. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J Allergy Clin Immunol* 2002;110:867-72.
60. Kline JN, Kitagaki K, Businga TR, Jain VV. Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L170-9.