

Animal shed *Bacillus licheniformis* spores possess allergy-protective as well as inflammatory properties

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Background: Numerous epidemiologic studies have demonstrated an allergy-protective effect of farm life early in childhood. It has been hypothesized that environmental exposure to microbes may contribute to this effect. Because of their small size and thereby their potential for deposition in lower airways of small children, bacterial spores may be candidates for such allergy-protective effects.

Objective: To investigate immune responses elicited by exposure to *Bacillus* spores in experimental settings.

Methods: Animal shed and mattress dusts were analyzed for bacteria and fungi by aerobic and anaerobic growth. *Bacillus licheniformis*, the most prominent microorganism found in these samples, was investigated with respect to spore specific stimulation of pattern recognition receptors, monocyte-derived dendritic cells and T_H-cell polarization *in vitro* as well as to the prevention of asthma development in a mouse model of allergic asthma.

Results: *In vitro*, *B. licheniformis* spores activated a T_H1 cytokine expression profile. *In vivo* application of these spores resulted in less spore-specific but long-lasting immune activation preventing eosinophilia and goblet cell hyperplasia; however, they provoked an influx of neutrophils in lung tissue of asthmatic mice.

Conclusion: Bacterial spores may contribute to the allergy-protective properties of farming environments, but their persistence in the lung causes ongoing immune activation in mouse experiments. (J Allergy Clin Immunol ■■■■; ■■■:■■■-■■■.)

Key words: Allergy protection, animal shed dust, hygiene hypothesis, *Bacillus licheniformis* spores, ovalbumin mouse model

Several epidemiologic investigations identified a protective effect of farm life with respect to asthma, hay fever, and atopic sensitization,^{1,2} which was related to a farming environment, high level exposure of microbial components, contact with farm animals, and gene-environment interactions.³ The risk of developing asthma was significantly reduced when contact with stables and thereby with animal sheds and feeding material such as hay occurred within the first year of life⁴ or even prenatally.⁵ In experimental studies, dust collected from cowsheds reduced airway inflammation and hyperresponsiveness in a mouse model of allergic asthma.⁶

A large variety of microbial exposures is present in stables and barns, but little is known about immune responses elicited by exposures to specific microbial components. Bacterial spores are small (about 1.5 μ m in diameter) and can therefore intrude into the lower airways even of young children. Thus, environmental exposure to bacterial spores may at least in part mediate the asthma and allergy protective effect. Sporulation enables such bacteria to survive extreme environmental conditions such as heat and dryness,⁷ including environments like animal shed dust.^{8,9} Furthermore, they are related to farm products like hay⁹ and are therefore present in high concentrations in the air of animal sheds, especially during littering. Moreover, endospore-forming bacteria such as *Bacillus* have been identified in dust samples from animal sheds and farm children's mattresses in Bavaria, south Germany.

It is conceivable that bacterial spores may germinate in the airways and stimulate the immune system not only as a spore but also as a vegetative bacterium. Therefore, the putative protective role of *Bacillus licheniformis* (strain designation, 467) was analyzed for both spores and vegetative cells. In addition, spores were heat-inactivated to abolish their capacity to germinate.¹⁰ An *in vitro* system of dendritic cells (DCs) and T cells as well as an *in vivo* mouse model of allergic asthma were used to assess the ability of *B. licheniformis* 467 spores (Blspo) and vegetative *B. licheniformis* 467 cells (vegBl) to counterregulate T_H2-driven immune responses¹¹ and the potential for prevention of the development of allergic asthma in mice.

METHODS

Dust sample collection

In a pilot study, dust samples from 24 dairy farms were collected in Bavaria in spring 2006. Samples were taken from 63 school-age farm children's mattresses according to Schram-Bijkerk et al¹² and from 36 animal sheds⁶ on those farms.

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Abbreviations used

BAL: Bronchoalveolar lavage
 Blspo: *Bacillus licheniformis* 467 spores
 DC: Dendritic cell
 hiBlspo: Heat-inactivated *Bacillus licheniformis* 467 spores
 MOI: Multiplicity of infection
 NOD: Nucleotide binding oligomerization domain
 TLR: Toll-like receptor
 vegBl: Vegetative *Bacillus licheniformis* 467 cells

The study was approved by the local ethics committee, and written informed consent was obtained from the children's parents.

Microbiological analyses

To obtain pure cultures from dust specimens, 10 mg of each sample were suspended in 190 μ L PBS. Dilutions of this suspension were spread on appropriate media. Three single colonies with typical appearance for *Bacillus* spp were subcultured and isolated for species identification by microscopic and biochemical methods as well as by the use of the API systems (Biomérieux, Germany). Fungi were identified macroscopically and microscopically, using sticky tape preparation with lactophenol blue staining.¹³

Isolation and strain characterization of *Bacillus licheniformis* 467

Bacillus licheniformis strain 467 was isolated on agar plates from a mattress of a farm child involved in this study. Strain characterization was performed following instructions by Relman et al¹⁴ with regard to 16S rDNA PCR and sequencing, and to Logan and Turnbull¹⁵ for further biochemical differentiation. Sequencing results of 16S rDNA were aligned to reference strain *B licheniformis* ATCC 14580 (GenBank entry CP000002) as published by Rey et al.¹⁶ Characteristic sequence similarities with exception of some changes also present in other *B licheniformis* strains were identified.

***Bacillus licheniformis* 467 growth and sporulation**

Bacillus licheniformis 467 was grown in LB medium (Lennox; Roth, Karlsruhe, Germany) at 37°C. vegBl were harvested after 6 hours of cultivation on a rotary shaker in the middle of the exponential growth phase, then washed twice with fresh LB medium to prevent sporulation, and finally lyophilized in aliquots.

Spore preparations were performed by cultivation of *B licheniformis* 467 in LB medium (Lennox) supplemented with 20 mg L⁻¹ MgSO₄ × 7 H₂O and 10 mg L⁻¹ MnSO₄ × H₂O (Merck, Darmstadt, Germany) on a rotary shaker for 60 hours and further sporulation for 3 weeks without shaking. Subsequently, Blspo were purified by heat-inactivation of vegetative cells at 80°C for 30 minutes, followed by density gradient centrifugation using 90% Percoll (GE Healthcare, Freiburg, Germany).¹⁷ Spores were washed 5 times with distilled water, and purity was controlled by Wirtz-Conklin staining¹⁵ (see this article's Methods in the Online Repository at www.jacionline.org). Preparations free of vegetative cells were used for *in vitro* and *in vivo* experiments. Heat-killed spores were obtained by steam sterilization (121°C; 20 minutes; 200 kPa).

Isolation of human PBMCs

Human PBMCs were isolated from citrate-buffered peripheral blood of different healthy donors, independent for each experiment, by gradient centrifugation using Ficoll (PAA Laboratories GmbH, Pasching, Austria),¹⁸ and stimulation with the respective form of *B licheniformis* 467 followed. When necessary, cells were separated by counterflow elutriation centrifugation to obtain monocytes and lymphocytes.

Human monocyte-derived DCs

Monocytes were isolated as described, concentrated to 10⁶ cells per mL, and differentiated into immature DCs in the presence of GM-CSF and IL-4

(500 U/mL each; Strathmann Biotec GmbH, Hamburg, Germany) by using RPMI (Cambrex, East Rutherford, NJ) medium containing 10% heat-inactivated FCS (Biochrom AG, Berlin, Germany), 100 U/mL penicillin (PAA Laboratories GmbH), and 100 μ g/mL streptomycin (PAA Laboratories GmbH). Cells were harvested after 5 to 7 days of the culture and stimulated at a multiplicity of infection (MOI) of 1 for bacteria and with 10 ng/mL LPS of *Salmonella enterica* sv Friedenau (kindly provided by Prof Dr Helmut Brade, Research Center Borstel, Germany), followed by the analysis of DC supernatants for the presence of TNF- α , IL-10, and IL-12 (eBioscience Human ELISA Ready-SET-Go! Kit; NatuTech, Frankfurt, Germany).

Mixed leukocyte reaction

Heterologous lymphocytes (2×10^5) were seeded into 96-well round-bottom plates (Nunc, Wiesbaden, Germany) in a total volume of 100 μ L per well; 2×10^4 , 2×10^3 , and 2×10^2 DCs, stimulated before mixed leukocyte reaction, were added to give final ratios of 1:10, 1:100, or 1:1000 DC:lymphocytes and incubated for 4 days. Cell-free culture supernatants were analyzed by Beadlyte Human Multi-Cytokine Detection System 4 (Upstate; Millipore Corp, Billerica, Mass). As a positive control, we used LPS of *S enterica* sv Friedenau, a strong stimulator of DC maturation,^{19,20} as well as *Mycobacterium bovis* BCG, inducing DC maturation without any T_H-cell polarization.²¹

Animals

Female BALB/c mice age 6 to 8 weeks from Harlan Winkelmann (Borchen, Germany) were kept under pathogen-free housing conditions. Water and an ovalbumin-free diet were supplied *ad libitum*. All experiments were performed according to federal and international guidelines and were accepted and controlled by local authorities (Regierungspräsidium Giessen).

Protocol of allergen provocation

Sensitization and challenge of mice to induce a local allergen provoked inflammatory response in the lungs was performed according to DeBarry et al.²² The detailed application is described in the protocol displayed in this article's Fig E1 in the Online Repository at www.jacionline.org. Intranasal treatment was started 12 days before the first ovalbumin injection, applying vegetative *B licheniformis* 467 cells in fresh LB medium to prevent sporulation, dormant spores, heat-inactivated *B licheniformis* 467 spores (hiBlspo) hindered in germination, or PBS as negative control over the whole process of sensitization and challenge.

Cell analysis of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) analysis was performed 48 hours after the last allergen challenge as described earlier.²³ Total leukocytes were determined with a Casy TT cell counter (Schaefer Systems, Reutlingen, Germany). Cytospin was performed at 300g, and cells were differentially stained with Diff Quick (Dade Diagnostics, Unterschleissheim, Germany). Remaining cell-free BAL fluids were stored at -20°C for further investigations of cytokine levels.

Detection of cytokines in BAL fluid

IFN- γ , IL-10, and IL-5 levels in BAL fluid were determined by using OptEIA ELISA kits (BD Biosciences, San Diego, Calif).

Lung histology

Lungs were fixed by infusion of a 4% (wt/vol) formaldehyde solution and stored in the same solution. Sections (3 μ m) of paraffin embedded lung tissues were stained with hematoxylin/eosin or periodic acid-Schiff.

Statistical analysis

All data are shown as means $\pm$ SEMs. Significance was analyzed by using Graph Prism software (Graph Pad Software, Inc, San Diego, Calif) and the Student unpaired *t* test. Significant differences were considered at *P* < .05.

For transmission electron microscopy, transfection of human embryonic kidney 293 cells, and airway responsiveness to methacholine, see Methods in the Online Repository.

RESULTS

Microbial flora of animal shed and mattress dusts

To investigate the bacterial composition of animal shed dusts, we analyzed 61 mattress dust and 36 animal shed dust samples of 24 traditional farms in Bavaria. Growth determinations on different agar plates for bacterial and fungal species indicated a great diversity (see this article's Table E1 in the Online Repository at www.jacionline.org). The most prominent microorganisms in settled dust from animal sheds and in mattress dusts were *Bacillus* spp and coliform bacteria. To investigate the transport of animal shed bacteria to the children's mattresses, colony-forming unit numbers in mattress and animal shed dusts were compared for all farms (Table E1). A correlation of colony numbers of all microorganisms in mattress and animal shed dust samples was calculated (see this article's Table E2 in the Online Repository at www.jacionline.org). The genera *Clostridium* and *Enterococcus* showed the highest correlation coefficients, but less than 1/3 of the children were in measurable contact with these microorganisms. Correlation for *Bacillus* spp, present in 97% of all mattress dust samples, was $r = 0.37$ ($P = .0039$). These findings support the idea of a relevant contact of farm children to *Bacillus* spores.

Detailed analysis of *Bacillus* spp in all collected dusts showed the presence of *B licheniformis* in 95% of all samples. In descending order, *Bacillus pumilus* (31%), *Bacillus subtilis* (28%), and *Bacillus cereus* (7%) followed with significantly reduced frequencies. Therefore, *B licheniformis* spores were chosen for further analyses. As expected, electron micrographs of these spores showed a particle size of about 1.5 μm (see this article's Fig E2 in the Online Repository at www.jacionline.org), which enables their inhalation into the lower airways, even in newborns.

Specific immune stimulation by *Bacillus licheniformis* 467 spores in a human *in vitro* system

To investigate whether Blspo as a dormant form of the bacterium was able to stimulate the innate immune system for T_H -cell polarization,²⁰ we analyzed the stimulatory capacity of *B licheniformis* spores on cytokine response of PBMCs. A differentiation between spore-specific results and stimulation by germinated bacteria was achieved by testing spores as well as vegetative cells and hiBlspo, which were no longer able to germinate. Blspo and hiBlspo caused a $\text{TNF-}\alpha$ release at comparable levels. In contrast, vegBl induced a significantly higher $\text{TNF-}\alpha$ release (Fig 1). Stronger cytokine expression by vegBl was furthermore achieved by stimulation of HEK293 cells transiently transfected with the pattern recognition receptors Toll-like receptor (TLR)-2 or peptidoglycan receptor nucleotide binding oligomerization domain (NOD)-2 (see this article's Table E3 in the Online Repository at www.jacionline.org).

No germination was microscopically observed in the *in vitro* medium (data not shown). On the basis of these spore-specific results, Blspo and vegBl were compared at an MOI of 1 in the following *in vitro* experiments.

Stimulation of monocyte-derived DCs

Next, dormant spores and vegetative cells were investigated concerning their ability to stimulate human DCs, important for

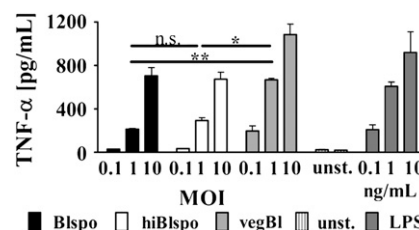


FIG 1. Stimulation of PBMCs by Blspo, hiBlspo, and vegBl. Data show means $\pm$ SEMs of $\text{TNF-}\alpha$ measurement in culture supernatant after dose-dependent (MOI) PBMC stimulation for 24 hours of 3 independent experiments from different healthy donors. * $P < .05$; ** $P < .01$. n.s., Not significant; unst., unstimulated.

differential immune responses to bacteria.²⁴ Therefore, the expression of a typical T_H1 -directing cytokine (IL-12p70), a proinflammatory cytokine ($\text{TNF-}\alpha$), and a regulatory cytokine (IL-10)²⁵ was analyzed (Fig 2, A). $\text{TNF-}\alpha$ expression and IL-10 expression were increased after stimulation with vegetative bacteria, in comparison with spores. Moreover, the strongest phenotypic maturation of human monocyte-derived DCs (moDCs), resulting in expression of costimulatory molecules such as CD83 and CD86, was achieved by the stimulation with vegetative bacteria (data not shown). In contrast, secretion of IL-12 was detectable exclusively after incubation with Blspo, indicating a spore-specific T_H1 signal.

Polarization of T_H cells

Because Blspo induced a spore-specific cytokine profile in DCs with a weak expression of cytokines, we analyzed the effect of stimulated DCs on T_H -cell polarization and the ability of Blspo to contribute to a counterregulation of allergic T_H2 polarization. As a characteristic cytokine for T_H1 -polarized cells, IFN- γ expression was measured. In contrast with IFN- γ , IL-5 and IL-13 were measured to determine cytokine expression of T_H2 cells responsible for the activation of eosinophils.²⁶ The strongest induction of IFN- γ was observed after maturation of DCs by *B licheniformis* 467 spores (Fig 2, B), whereas IL-5 and IL-13 were not released. In contrast, IL-5 and IL-13 were induced by stimulation with vegetative bacteria.

Effect of intranasal *B licheniformis* pretreatment on asthma induction in mice

To investigate whether the specific immune stimulation found for *B licheniformis* 467 spores *in vitro* was able to prevent the establishment of allergic asthma *in vivo*, we applied a protocol to induce experimental asthma (Fig E1). Spore-specific effects were analyzed by comparison of those spores able to germinate to those hindered in germination by heat-inactivation, as well as to vegetative cells. Analyses of BAL cells showed a significantly decreased number of eosinophils (T_H2 triggered effectors) in all *B licheniformis*-treated groups in contrast with the sham-treated mice (Fig 3, A). Application of vegBl resulted in the strongest reduction of eosinophils with significant differences to the hiBlspo-treated group. On the contrary, increased neutrophil numbers (T_H1 triggered effector cells) were confined to treatment with vegBl and spores able to germinate (Fig 3, B).²⁶ In Blspo-treated groups, cell numbers of eosinophils and neutrophils were in between those scores found for mice treated with either vegBl or hiBlspo, indicating ongoing germination. The decrease of lymphocytes after spore treatment showed that spores but not vegBl

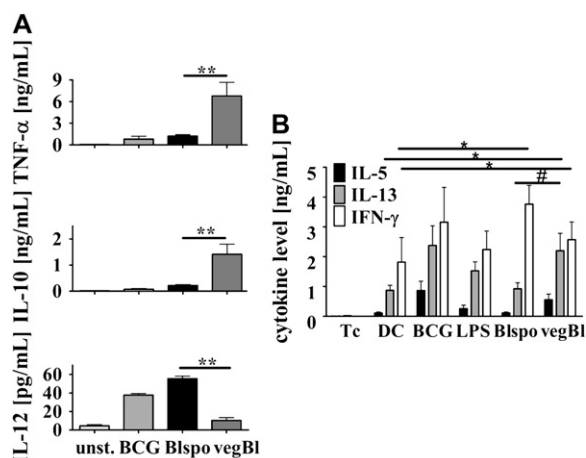


FIG 2. Stimulation of moDCs by *Bacillus licheniformis* 467 (MOI = 1) for 24 hours (A) before mixed leukocyte reaction (B). Cells were isolated from peripheral blood of healthy donors. Results of cytokine measurements are expressed as the mean of 3 independent experiments $\pm$ SEM. BCG, *Mycobacterium bovis*; DC, unstimulated DCs plus leukocytes; LPS, LPS 10 ng/mL; TC, leukocytes alone; unst., unstimulated.

were able to downregulate the specific immune reaction (Fig 3, C) caused by ovalbumin sensitization and challenge.

Lung histology revealed a decrease of peribronchial inflammation and an almost complete prevention of goblet cell hyperplasia by pretreatment with the bacterium in all its forms (Fig 4, A). Despite this observation, airway hyperreactivity to methacholine was only marginally improved by bacterial administration (see this article's Fig E3 in the Online Repository at www.jaci.org). Contrary to this beneficial effect, lung histology showed the formation of multinucleated giant cells by macrophage fusions as a sign for ongoing bacterial clearance in the lung (Fig 4, B).²⁷ This was supported by the increased infiltration of macrophages in the BAL after spore treatment (Fig 3, D).

The cytokine level in the BAL was investigated to clarify the state of inflammation in the airways. Spore treatment led to a 50 $\times$ increase of IFN- γ levels in comparison with sham-treated mice, corresponding with the observed high number of neutrophils in the BAL (Fig 5). On the other hand, hiBlspo and vegBl caused only marginal induction of IFN- γ and IL-10.

To confirm whether the high cytokine levels were caused by an ongoing immune activation, we analyzed lung tissue and BAL cytopins for the presence of Blspo and vegBl. Wirtz-Conklin staining enabled the detection of *B licheniformis* 467 spores in alveolar macrophages independent of heat-inactivation (see this article's Fig E4 in the Online Repository at www.jacionline.org). Vegetative bacteria, on the other hand, could not be detected by any staining method applied, irrespective of a previous treatment with vegetative bacteria or with spores competent in germination. Outside macrophages, neither spores nor vegetative bacteria were found (Fig E4). In this way, even 4 days after the last treatment, an ongoing immune activation was demonstrated by the presence of high cytokine levels and the formation of multinucleated giant cells. The T_H2 cytokine IL-5, responsible for activation of eosinophils, was not significantly reduced in the absence of eosinophils.

DISCUSSION

A clear protective effect of both cowshed dust extract and certain bacteria on the establishment of experimental asthma was shown in recent publications.^{6,22} The basis of our investigations

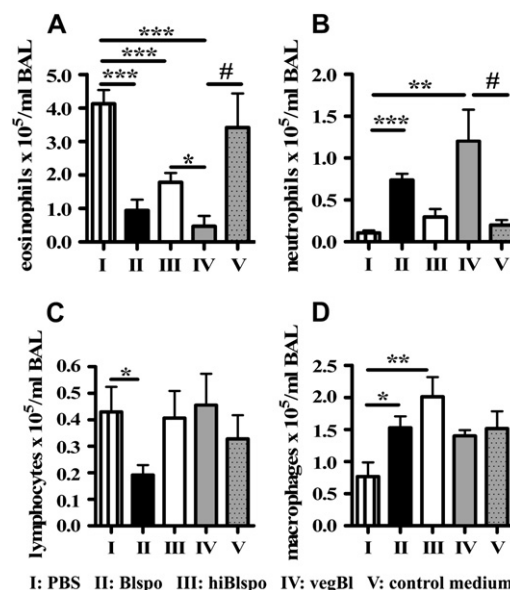


FIG 3. Effect of intranasal treatment with *Bacillus licheniformis* 467 on the airway inflammation of ovalbumin-sensitized and challenged mice (n = 8). Mice BAL fluid was assessed for numbers of total eosinophils (A), neutrophils (B), lymphocytes (C), and macrophages (D). Depicted are means $\pm$ SEMs; significant differences between sham-treated (PBS) mice and bacteria treated mice are indicated by * P < .05; ** P < .01; *** P < .001. # indicates significant differences between vegBl-treated mice and control medium-treated mice.

was a detailed analysis of the microbial composition of protective cowshed dusts. We identified a great diversity of microbial species in cowshed and mattress dusts from 24 dairy farms. The genus *Bacillus* showed high colony-forming unit numbers and a significant correlation between the presence in farm children's mattresses and animal sheds on these farms for almost all children involved in the study. Thus, farm children are likely to be exposed to *Bacillus* spp to a substantial extent. The predominant *Bacillus* species was *B licheniformis*, and the isolated strain 467 was investigated in detail, in particular concerning its allergy-protective properties.

The essential role of antigen-presenting cells such as DCs, which link innate and adaptive immunity, consists in its effect on T-cell development.²⁸ DC stimulation by *B licheniformis* 467 mediated by TLR2 and NOD2, as shown for transfected HEK cells, resulted in a spore-specific IL-12 induction after spore stimulation, in contrast with an IL-10 and TNF- α -dominated cytokine expression profile for vegBl. Furthermore, stimulation with these different *B licheniformis* forms led to significant differences in T_H-cell polarization. *In vitro* stimulation with Blspo but not with vegBl resulted in upregulation of the T_H1 cytokine IFN- γ , which itself is known to downregulate T_H2 cytokines.^{29,30} Vegetative cells, in contrast, induced T_H2 cytokine expression (IL-5 and IL-13). These results led to the question whether spore pretreatment could prevent the establishment of allergy-related T_H2 immune responses *in vivo*.

Comparing BAL eosinophil, neutrophil, and macrophage subpopulations after treatment with Blspo of ovalbumin-sensitized mice, the fluid cell numbers were intermediate with respect to vegBl and hiBlspo, indicating spore germination in the airways. A measurement of BAL cytokines supported this hypothesis, because high concentrations of IFN- γ and IL-10 were observed exclusively in mice treated with Blspo able to germinate. In both mice groups treated either with Blspo or with hiBlspo, spores

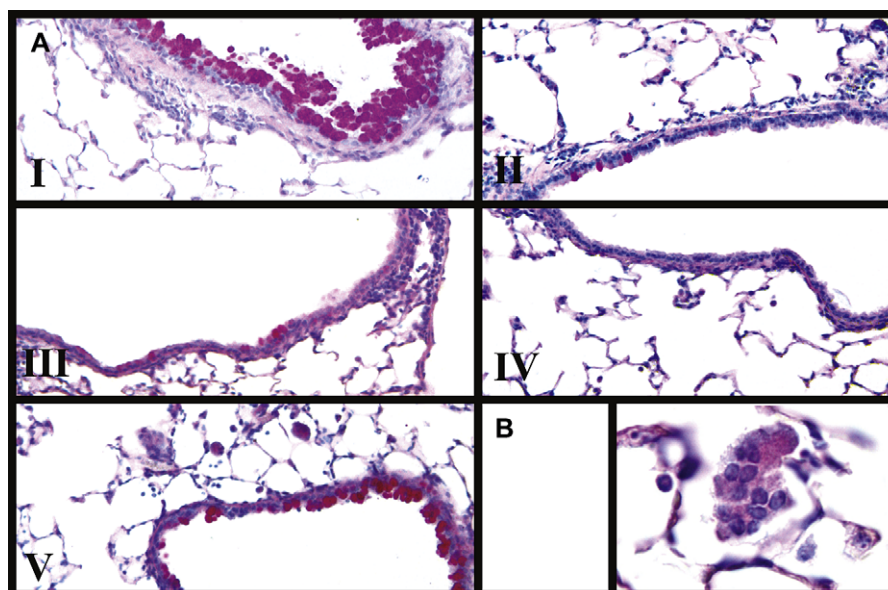


FIG. 4. Prevention of goblet cell hyperplasia (**A**) and fusion of mononuclear cells (**B**) by *Bacillus licheniformis* 467 treatment. Shown are representative micrographs of mice airways after periodic acid-Schiff staining. **A**, I, sham-treated mice; II, Blspo-treated mice; III, hiBlspo-treated mice; IV, vegBl-treated mice; V, control medium-treated mice. **B**, Formation of multinucleated giant cells shown is representative of all *B. licheniformis*-treated groups.

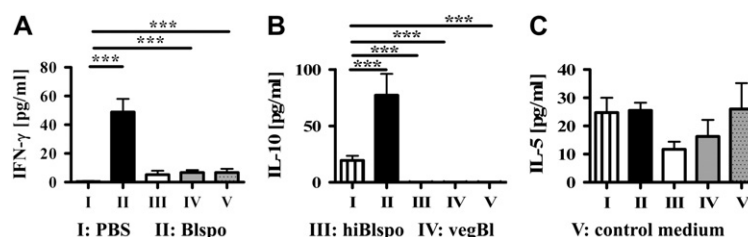


FIG. 5. BAL cytokines IFN- γ (**A**), IL-10 (**B**), and IL-5 (**C**) measured after *B. licheniformis* 467 treatment of mice sensitized and challenged to ovalbumin ($n = 8$). The determination of cytokine levels was performed by ELISA of BAL fluid. Shown are results as means $\pm$ SEMs for each treated group. Significant differences between sensitized PBS-treated and bacteria-treated mice are indicated by * $P < .05$; ** $P < .01$; *** $P < .001$.

were observed in the lung tissue, phagocytosed by increased numbers of alveolar macrophages, even 4 days after the last treatment. However, germination was not demonstrated directly by staining for vegBl. In this way, the *in vivo* results for Blspo-treated mice were not as spore-specific as they were *in vitro*.

IFN- γ is expressed by T_H1 cells to activate cellular defense mechanisms, namely macrophage and neutrophil recruitment. In this case, a direct association in the numbers of neutrophils and macrophages would be expected.³¹ The inverse association we found, with high neutrophil numbers in BAL of mice treated with vegBl and high macrophage numbers in BAL of Blspo-treated mice, indicated 2 different mechanisms of clearance of these cell forms in the lung: on the one hand, a fast clearance of vegBl by neutrophils, and on the other hand, a clearance of spores by macrophages.³² Intermediate results for Blspo indicate slow germination of single spores, activating a moderate but long-lasting immune response. This is supported by the fact that increased IFN- γ levels were measured only in the spore-treated groups. Thus, eosinophilic inflammation in the airways was prevented by shift from a T_H2 triggered allergic response to a process characterized by neutrophil infiltration. Neutrophils are recruited on IL-12 signal by DCs,²⁸ $CD8^+$ T cells, natural killer cells,³³ or

T_H17 cells.²⁶ Other bacteria such as *Acinetobacter lwoffii* or *Lactococcus lactis*²² have been reported to downregulate eosinophilic infiltrations without inducing neutrophil infiltration. This was accompanied by high IL-12 levels after stimulation of moDCs. Thus, through which mechanisms Blspo and vegBl cause neutrophil infiltration must be investigated further.

Bacillus licheniformis 467 spores and vegBl lead to an almost complete absence of mucus-producing goblet cells despite provocation by ovalbumin sensitization and challenge. However, this was not related to airway hyperreactivity. Different pathways may be responsible for this phenomenon: improvement of airway hyperreactivity has been reported to be IL-12-dependent, whereas eosinophilic infiltrations resulted from neutralization of IL-10.³⁴ Because high expression levels of IL-10 were detected in the current study after stimulation of moDCs *in vitro* and in BAL fluid, our data are in line with a beneficial effect of IL-10 on the prevention of eosinophilia. In contrast, beneficial effects of IL-12 were not observed, presumably because of low expression levels *in vitro*. With respect to the effects of *Acinetobacter lwoffii* and *Lactococcus lactis*,²² a higher induction of IL-12 expression by moDCs is necessary to reduce airway hyperreactivity. In an inflammatory state, regulatory T cells, induced by IL-10

expression of DC,²⁵ secrete high levels of IL-10, which is acting as an immunosuppressive agent³⁵ and facilitates the clearance of *B licheniformis* in the lung in an IL-12-independent way.

In summary, *B licheniformis* 467 spores did not germinate *in vitro*, leading to a spore-specific T_H1-polarizing cytokine pattern. *In vivo* intranasal application of *B licheniformis* 467 resulted in decreased eosinophilia and a reduction of mucus-producing goblet cells on one side, but to neutrophil infiltration, provoking a T_H1-type inflammation on the other side. In the context of potential asthma prevention by inhalation of spores into the lower airways, the study clarified that there is an effect on the reduction of eosinophilic infiltrations, but not on the improvement of airway hyper-reactivity. However, the *in vitro* spore-specific effects directing to a T_H1 polarization dependent on IL-12 expression were not verified *in vivo*. Inhalation of vegB1 was cleared by neutrophil recruitment, whereas the clearance of spores was processed by accumulated macrophages. In this way, germination of Blspo in the lung led to a delayed but long-lasting immune response.

The aim of the study was to investigate the contribution of bacterial spores to the allergy-protective effect of the animal shed environment, and we could clearly show some immunostimulatory activity of *Bacillus* spores. It is possible that exposure to a wider range of farm bacteria results in a synergistic effect as indicated by earlier investigations on *Acinetobacter lwoffii* and *Lactococcus lactis*; however, this has not been proven to date.

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Clinical implications: This study provides insight into the response to inhaled microbial spores and their potential impact in allergy-protective effects of farm environments for prospective applications of isolated microbial components.

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METHODS

Wirtz-Conklin stain of spores

Bacterial suspensions were prepared on an object slide, heat-fixed, and covered with 5% aqueous malachite green (Merck, Darmstadt, Germany). The covered slide was heated gently to steaming for 5 minutes to enhance dye uptake by bacterial spores, rinsed with water, and counterstained for 1 minute with 1% aqueous safranin (Merck, Darmstadt, Germany). Following safranin removal by washing, the dried slide was examined by light microscopy.

Transmission electron microscopy

Spores and vegetative bacteria were prepared as described in the article. Transmission electron microscopy was performed according to Andrä et al.^{E1} Spores were washed 5 times in distilled water, whereas vegetative bacteria were washed 3 times in PBS. The pellets were fixed in 2% osmium tetroxide for 1.5 hours and washed 3 times with distilled water. Postfixation positive stain was followed by addition of 2% aqueous uranyl-acetate and incubation at 25°C for 1 hour. After washing, the pellet was dehydrated in serial ethanol washes. After the transfer to propylene oxide for 15 minutes, samples were infiltrated at 4°C for 16 hours by a 1:1 mixture of propylene oxide-Epon-resin mix, (Serva, Feinbiochemica, Heidelberg, Germany), and polymerization followed embedding in an Epon-resin-mix at 60°C for 16 hours. Ultrathin-sections were cut by using a paramyotom. Slices were placed on a copper grid and counterstained in lead citrate in a CO₂-free environment, and micrographs were taken with an EM Zeiss 910 (Carl Zeiss AG, Oberkochen, Germany).

Transfection of HEK293 cells

HEK293 cells were transfected with the expression plasmids for TLR2, TLR4, NOD1, and NOD2.^{E2} Twenty-four hours postinfection, cells were washed with PBS and stimulated with either live or lyophilized vegetative

cells of *B. licheniformis* 467 spores or heat-inactivated spores for 18 hours. Concentrations of IL-8 in cell-free supernatant were determined by ELISA (Biosource, Solingen, Germany). As positive controls, Pam₃CSK₄ (EMC Microcollection, Tübingen, Germany) and muramyl dipeptide,^{E3} kindly provided by Prof Koichi Fukase (Osaka, Japan), were used.

Airway responsiveness to methacholine

Midexpiratory airflow was measured by head-out body plethysmography as described previously,^{E4} and the concentration of β -methacholine responsible for a 50% reduction in midexpiratory airflow was reported.

Statistical analysis

All data are shown as means $\pm$ SEMs. Significance was analyzed by using Graph Prism software (Graph Pad Software, Inc, San Diego, Calif) and the Student unpaired *t* test.

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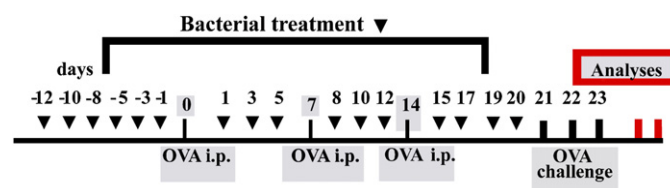


FIG E1. Schematic protocol of the experimental design for mouse experiments. Female BALB/c mice were sensitized at days 0, 7, and 14 by ovalbumin (OVA) intraperitoneal (*i.p.*) injections followed by OVA aerosol exposure at days 19, 20, and 21. Twelve days before the first OVA injection and during the whole sensitization period, mice received *B licheniformis* 467 spores, heat-inactivated *B licheniformis* 467 spores, or vegetative *B licheniformis* 467 intranasally every second day. Mice were analyzed at days 22 and 23 for airway inflammation and lung function.

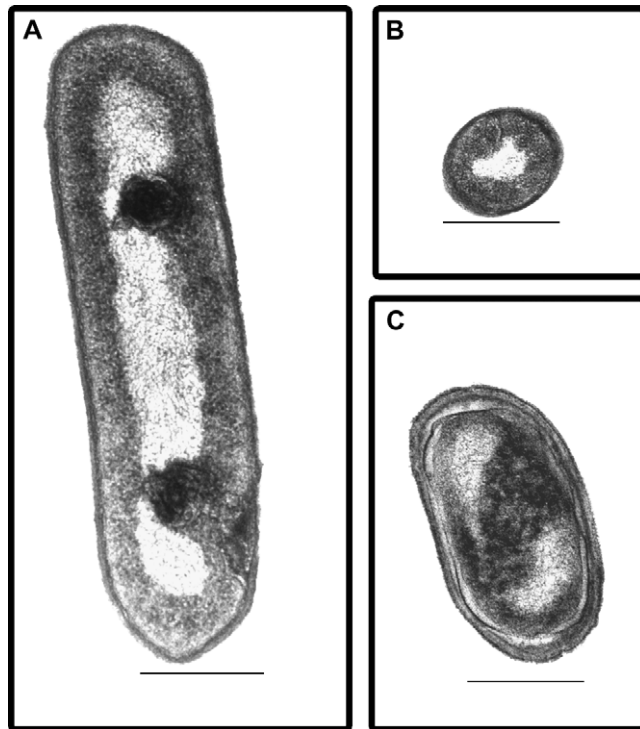


FIG E2. Transmission electron micrograph of *B. licheniformis* 467. The vegetative bacterium, obtained from the log-phase of growth, is shown in longitudinal (**A**) and cross (**B**) sections. **C**, The dormant spore protected by several layers of highly cross-linked proteins and peptidoglycan. Bars indicate 0.5 μm .

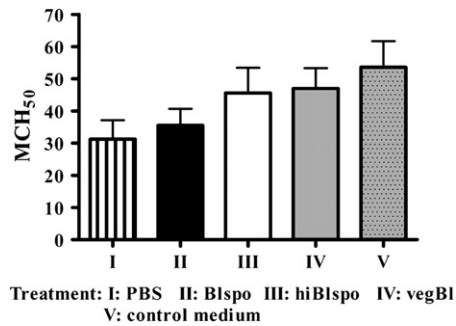


FIG E3. Analyses of lung function by head-out body plethysmography. Mice sensitized and challenged to ovalbumin, during and after bacterial treatment with *B licheniformis* 467 as the indicated forms. Methacholine concentrations that caused 50% reduction in baseline midexpiratory airflow (MCH_{50}) are displayed. No treatment was able to achieve significant increase in airway hyperreactivity.

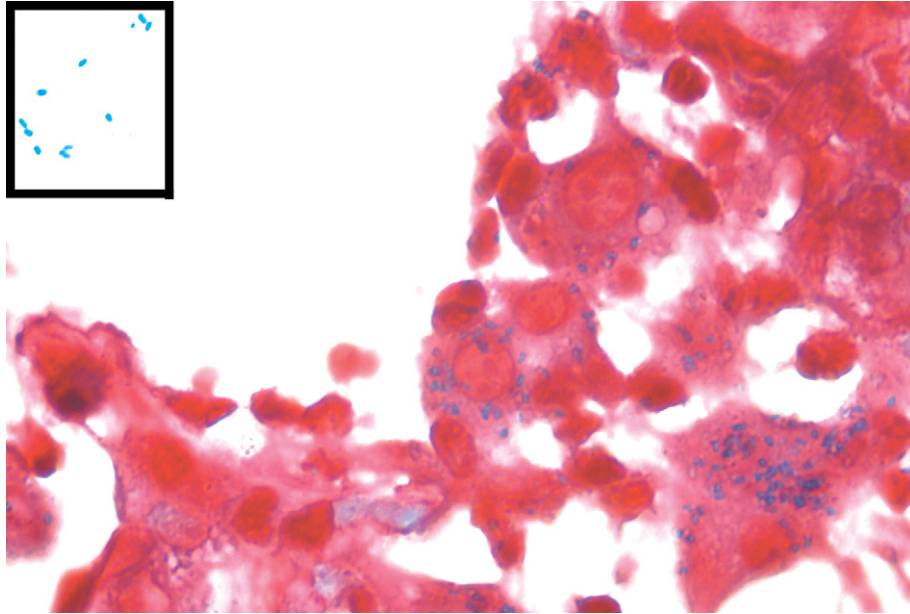


FIG E4. Lung tissue of *B licheniformis* 467 spore-treated mice after Wirtz-Conklin staining phagocytosed by alveolar macrophages. Staining was performed by using paraffin-embedded lung tissue 4 days after the last spore treatment. *Left upper corner* shows *B licheniformis* spores on an object slide. Magnification $\times 1000$. Similar results were obtained by staining lung cytopspins of Blspo and hiBlspo.

TABLE E1. Analyses of the microbial flora of cowshed dusts and farm children's mattresses from 24 Bavarian farms (shed/mattress)

Isolated microorganisms	Median/IQR (log)		Shed/mattress			
	mattress dust	Shed dust	+/+	+/-	-/+	-/-
<i>Bacillus</i> spp	5.25/0.87	6.26/0.84	24	0	0	0
Coliform bacteria	5.67/0.94	5.83/0.77	24	0	0	0
<i>Lactobacillus</i> spp	3.00/3.30	3.00/3.78	9	7	5	3
<i>Enterococcus</i> spp	3.15/4.47	3.48/3.95	11	7	3	3
<i>Clostridium</i> spp	0.00/3.00	3.00/3.78	9	6	3	6
<i>Aspergillus</i> spp	4.00/1.10	4.88/0.93	23	1	0	0
<i>Mucor</i> spp	3.75/1.00	4.48/1.15	21	2	1	0
<i>Cladosporium</i> spp	0.00/3.74	4.00/5.34	10	4	3	7
<i>Penicillium</i> spp	3.00/4.00	0.00/3.95	8	3	9	4
<i>Wallemia</i> spp	3.48/4.00	4.70/5.35	16	3	4	1

Median logarithmic values of colony-forming units/interquartile range (*IQR*) are shown. Moreover, concordant and discordant results between sheds and children's mattresses on these farms clustered for 24 families are given for the respective microorganisms.

TABLE E2. Correlation of colony numbers of all microorganisms in mattress and animal shed dust samples

Microorganism	N*	Partial correlation coefficient†	P value
<i>Clostridium</i> spp	16	0.55	.0340
<i>Enterococcus</i> spp	20	0.54	.0181
<i>Bacillus</i> spp	61	0.37	.0039
<i>Cladosporium</i> spp	16	0.34	.2212
<i>Wallemia</i> spp	30	0.31	.0990
Anaerobic bacteria	57	0.23	.0841
<i>Mucor</i> spp	48	0.21	.1547
Aerobic bacteria	58	0.19	.1574
<i>Penicillium</i> spp	14	0.14	.6389
<i>Lactobacillus</i> spp	16	0.06	.8271
<i>Aspergillus</i> spp	55	−0.02	.8666
Coliform bacteria	61	−0.17	.2063

The genus investigated in more detail in this study is in boldface text.

*Number of children with measurable exposure to the respective microorganisms in animal sheds.

†Partial correlations are adjusted for family clusters.

TABLE E3. Expression of IL-8 (pg/mL) of transiently transfected HEK293 cells stimulated for 24 hours with *B licheniformis* 467 preparations at a MOI = 1

HEK 293 cells expressing	Blspo	hiBlspo	vegBl	Unstimulated	Positive control*
TLR 2	1245.5 ± 345.1	1253.2 ± 185.47	12,365.5 ± 2273.3	8.2 ± 4.7	5234.5 ± 477.2
NOD2	305.0 ± 102.1	193.5 ± 7.5	1015.4 ± 81.9	156.3 ± 31.5	1103.6 ± 57.6

Vegetative bacteria show higher activation of transfected HEK293 cells than spores do.

*As a positive control for stimulation of TLR2-transfected HEK293 cells, Pam₃CSK₄—^{E2} was used. Positive control for the stimulation of NOD2-transfected cells was muramyl dipeptide.^{E3}