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Intranasal Immunization with a Colloid-Formulated Bacterial Extract Induces an Acute Inflammatory Response in the Lungs and Elicits Specific Immune Responses

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Nonspecific stimulation of lung defenses by repeated oral administration of immunomodulators, such as bacterial extracts, has shown potential for the prevention of respiratory tract infections. Here, we show that intranasal (i.n.) immunization with a bacterial extract formulated as a colloid induces an acute inflammatory response in the lungs characterized by increased production of CCL and CXCL chemokines and a major influx of dendritic cells (DCs) and neutrophils, with a higher proportion of DCs showing an activated phenotype (high CD80/CD86 expression). Cytokine levels measured in bronchoalveolar-lavage samples showed a small increase in the production of tumor necrosis factor alpha and similar levels of the other cytokines measured (interleukin 10 [IL-10], IL-12, and gamma interferon [IFN-γ]) in immunized mice compared with control mice. However, the recall response of primed animals after antigenic challenge induced increased expression of IL-12 and IFN-γ mRNAs in lung homogenates. Overall, all these effects were not due to the lipopolysaccharide content in the bacterial extract. Furthermore, we found that three i.n. doses administered 2 to 3 weeks apart were enough to elicit long-lasting specific serum immunoglobulin G (IgG) and secretory IgA antibody responses. Assessment of IgG subclasses showed a balanced pattern of IgG1-IgG2a responses. The serum total IgE concentrations were also elevated in immunized mice 2 weeks after the third dose, but they significantly decreased soon afterwards. Our results suggest that simple formulations of bacterial extracts administered i.n. are highly immunogenic, eliciting local and systemic immune responses, and may serve as the basis for cost-effective immunotherapies for the prevention and treatment of respiratory infections.

Respiratory tract infections (RTI) are among the most frequent infections in humans, with major socioeconomic impact (3, 16, 23). *Streptococcus pneumoniae* is still one of the main bacterial causes of RTI and is responsible for 10 million deaths worldwide per year (73). The introduction of a conjugate vaccine has the potential to change this situation, although economic factors may hamper the massive use of these vaccines in developing countries. Other bacterial agents of RTI and otitis media in children include nontypeable *Haemophilus influenzae* and, to a lesser extent, *Moraxella catarrhalis* (4, 9, 24, 47). On the other hand, recurrent RTI in the elderly, immunocompromised people, and patients with respiratory base pathologies involve these and other agents (e.g., *Klebsiella pneumoniae*) and are generally associated with a reduced defense capacity of the respiratory mucosa (2, 25, 38, 62, 63, 71). The prevention and treatment of these pathologies remain major health care challenges for developed and underdeveloped countries.

An alternative measure, which has been claimed to have good potential and has been in use for many years for the prevention and treatment of RTI, consists of the nonspecific stimulation of lung defenses by repeated oral administration of immunomodulators, mainly bacterial extracts (BEs). Such preparations stimulate various nonspecific humoral and cellular immune defense mechanisms (7, 8, 40, 41, 50, 75), and it has been suggested that they may also stimulate specific immune responses, although such an effect has been observed only after repeated administration for long periods of time (a daily dose over 300 consecutive days) (31). Although not fully characterized, these BEs have been tested in several clinical trials, including double-blind randomized studies with the use of a placebo, and have been shown to produce therapeutic benefits for patients (20, 33, 54, 68). It has also been demonstrated that they can reduce the number of respiratory infections in patients prone to repeated infections, including children, adults, and elderly people (15, 18, 48, 52, 55, 76), and they proved effective in patients with chronic obstructive pulmonary disease (14, 48).

Immunomodulatory BEs are commonly administered by the oral route, and it has been proposed that they exert their effects on lung defenses by specific cell trafficking through the common mucosal immune system (23). However, accumulated evidence suggests that direct stimulation of lung immune responses via the intranasal (i.n.) route would be more effective (70), as there are nasal, bronchoalveolar, and larynx-associated lymphoid tissues with a high density of mucosal dendritic cells

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TABLE 1. Sequences of murine primers used for RT-PCR

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	TAA AAC GCA GCT CAG TAA CAG TCC G	349
KC (CXCL1)	CC TTG ACC CTG AAG CTC CCT TGG TTC	CGT GCG TGT TGA CCA TAC AAT ATG	506
MIP-2 (CXCL2)	CCC CCT GGT TCA GAA AAT CAT CC	TCC CCA GTC TCT TTC ACT GT	465
MIG (CXCL9)	GAT CAA ACC TGC CTA GAT CC	GGC TGT GTA GAA CAC AGA GT	399
IP-10 (CXCL10)	TCG CAC CTC CAC ATA GCT TAC AG	TCA GCA GAG ATG TCT GAA TC	382
MIP-1α (CCL3)	CCG GAA GAT TCC ACG CCA ATT C	TGA GGA ACG TGT CCT GAA G	427
MIP-1β (CCL4)	CCC ACT TCC TGC TGT TTC TCT TAC	AGC AGA GAA ACA GCA ATG GTG G	427
IL-1	GGA AGA TTC TGA AGA AGA GAC GG	TGC GAT TTT TAG AGT AAC AGG	329
TNF-α	GAT CTC AAA GAC AAC CAA CAT GTG	CTC CAG CTG GAA GAC TCC TCC CAG	255
IFN-γ	GCA TCT TGG CTT TGC AGC T	CCT TTT TCG CCT TGC TGT TG	316
IL-12	AGA CCA GAG ACA TGG AGT CAT A	TGC TTC ACA CTT CAG GAA AG	205

(DCs) that form a contiguous network throughout the airway epithelium, placing them in an ideal position to sample and process inhaled antigens (26, 59). The i.n. route is usually more effective than the oral route for induction of mucosal immune responses, eliciting stronger responses in the lungs while also producing immune responses at distal sites, such as the intestine, vagina, and rectum (35, 51, 70).

On the other hand, it has been reported that local challenge of the airway mucosa with heat-inactivated bacteria (installed directly in the trachea via surgical intervention or applied as an aerosol) results in the immediate recruitment of fresh DCs in large numbers that peak within 24 h after the challenge and return to basal levels in the next couple of days (44, 45).

In this work, we explore the i.n. route as an alternative for administration of BEs, using a colloid-formulated BE containing soluble components of several gram-positive and gram-negative bacteria frequently implicated in respiratory infections. We characterize the proinflammatory response induced in the lungs and assess the development of specific immune responses against the bacteria included in the preparation.

MATERIALS AND METHODS

BEs. The BE (IRASA Laboratory, Montevideo, Uruguay) is a soluble extract of several pathogens frequently implicated in respiratory infections (*S. pneumoniae*, *M. catarrhalis*, *H. influenzae*, *K. pneumoniae*, *Staphylococcus* sp., and *Streptococcus* sp.). The total protein concentration of the extract was estimated to be 80 μg/ml by the bicinchoninic acid method (Sigma, St. Louis, Mo.). A colloidal formulation of the extract (CBE; IRASA Laboratory) is presently registered and marketed in Uruguay as a nasal immunostimulant for children and is formulated by adding a 1:5 dilution of the original BE to a gelatin-stabilized silver iodide colloid.

The endotoxin concentrations in BE and CBE samples were estimated through the *Limulus* amebocyte lysate test (Associates of Cape Cod Inc., Falmouth, Mass.) used according to Food and Drug Administration guidelines and the manufacturer's instructions. The lipopolysaccharide (LPS) contents were estimated at 25.00 and 1.25 ng/ml for the BE and CBE, respectively.

I.n. immunization model. Female C57BL/6 mice (6 to 8 weeks old; Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. For i.n. immunization, the mice were lightly anesthetized and 15 μl of CBE or phosphate-buffered saline (PBS) was introduced dropwise into each nostril. The animals were kept in a vertical position for 1 min to ensure migration of the inoculum to the alveoli.

BAL. Mice were sacrificed by cervical dislocation and dissected so that the trachea was exposed. A fine-tip pipette was then inserted into a small nick in the trachea, and 1.0 ml of PBS containing 10 mM EDTA was repeatedly flushed into the lungs. Bronchoalveolar lavage (BAL) fluids were collected in tubes previously treated with PBS–0.1% bovine serum albumin (BSA) and centrifuged at 3,400 × *g* for 5 min, and the supernatants were conserved at −80°C until they were used. The cell pellets were resuspended in PBS, and cell populations were enumerated from May-Grunwald Giemsa-stained cytospin preparations.

Preparation of lung single-cell suspensions. Lung single-cell suspensions were prepared for flow cytometry (FC) analysis. Mice were sacrificed, and the pulmonary and systemic circulation was perfused with saline-EDTA to remove the intravascular pool of cells. BAL samples were prepared as described above, and then the lungs were carefully separated from the thymic and cardiovascular remnants and thoroughly minced using iridectomy scissors. Lung pieces were collected and incubated in RPMI containing 1.4 mg of type IX collagenase (Sigma)/ml and 30 μg of type I DNase (Sigma)/ml at 37°C and 5% CO_2 for 40 min. The lung pieces were then mashed through sterile metal screens in culture medium, and the resultant suspension was filtered through two layers of nylon membrane. The cells were washed with PBS containing 10 mM EDTA and finally resuspended in PBS containing 5% fetal bovine serum (Gibco BRL, Life Technologies, New York, N.Y.) and 10 mM EDTA. The cells were counted on a Coulter hematocytometer (ABX Diagnostics, Montpellier, France).

FC analysis of T cells and DCs in the lung. Local recruitment of DCs was evaluated by FC immunophenotypic analysis of lung single-cell suspensions prepared as described above. Briefly, 10^6 cells were incubated with the following antibodies: phycoerythrin (PE)-conjugated anti-IA^b, fluorescein isothiocyanate (FITC)-conjugated anti-CD80 or anti-CD86, and biotinylated anti-CD11c (all reagents were supplied by BD Pharmingen, San Diego, Calif.). For T-cell analysis, 10^6 cells were incubated with the following antibodies: PE-conjugated anti-CD8, FITC-conjugated anti-CD4, and biotinylated anti-CD3 (all from BD Pharmingen). After two washes, the cells were incubated with Cychrome5-conjugated strepatvidin (BD Pharmingen) for 15 min. The cells were washed twice and resuspended in 500 μl of IsotonII (BD Pharmingen) before being analyzed. All antibody incubations were performed for 15 min at room temperature in the dark, and 2% pooled mouse serum was added to the PBS buffer to avoid nonspecific binding to Fc receptors. Optimal antibody concentrations were previously defined by titration. FC data were collected on a FACSCalibur (BD Pharmingen) equipped with a single argon laser tuned at 488 nm. For data acquisition and analysis, the CellQuest software package (BD Pharmingen) was used. Positivity for DCs was established through a multidimensional gating strategy in which forward and side scatter and specific fluorescence staining-associated parameters, such as CD11c and major histocompatibility (MHC) class II, were included.

Isolation and RT-PCR amplification of whole-lung mRNA. Whole lungs were harvested, immediately immersed in TRizol (Life Technologies, New York, N.Y.), and stored at −80°C until they were processed for total RNA isolation according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed as previously described (64). Briefly, lung tissue was homogenized in 1 ml of TRizol, followed by chloroform extraction and ethanol precipitation. Air-dried RNA pellets were dissolved in diethylpyrocarbonate-treated water, and single-stranded cDNAs were synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.) according to the supplier's specifications. PCRs were performed using *Taq* polymerase (Life Technologies Inc.) in a PTC-200 thermocycler (MJ Research, Waterton, Mass.). The pairs of primers and the expected sizes of PCR products are shown in Table 1. The reaction conditions were 27 cycles of 3 min at 95°C, 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, with a final extension step of 7 min at 72°C. β-Actin expression on each cDNA sample was used as an internal standard for

the amount of mRNA. PCR products were separated by acrylamide gel electrophoresis and stained with silver nitrate.

ELISA determination of cytokines in BAL samples. Cytokine production was evaluated in BAL samples from the same groups of mice used for FC analysis. Gamma interferon (IFN-γ), interleukin 10 (IL-10), and bioactive IL-12 (p70) were assayed by enzyme-linked immunosorbent assay (ELISA) using a pair of specific monoclonal antibodies (capture and detection) against each cytokine and dilutions of the recombinant cytokine for the construction of standard curves (all reagents were supplied by BD Pharmingen as previously described (13). Mouse tumor necrosis factor alpha (TNF-α) in BAL samples was assayed using the ELISA Biotrack System (Amersham Biosciences United Kingdom Ltd., Little Chalfont, England), according to the manufacturer's instructions.

Antibody responses. Antibody responses were evaluated in serum and in BAL samples. Mice were immunized on days 1, 25, 39, and 110, and tail blood was collected prior to the first immunization (day 0) and on days 24, 38, 54, 110, and 120 after priming. The mice were sacrificed on day 120 to obtain BAL samples.

Antibody responses were assessed by ELISA as previously described (12). Briefly, 96-well plates (Maxisorp; Nunc, Naperville, Ill.) were coated with 50 μl of a solution of 6 μg of BE/ml diluted in 0.1 M carbonate buffer (pH 9.6)/well. The plates were blocked with PBS–1% BSA (ICN Biomedical Inc., Aurora, Ohio) for 1 h. After being washed with PBS– 0.05% Tween (PBS-T), individual sera were added at an appropriate dilution in PBS-T– 0.1% BSA (PBS-T– BSA). Biotinylated goat anti-mouse immunoglobulin G (IgG), IgG1, IgG2a, IgA, or IgE (Southern Biotechnology Associates Inc., Birmingham, Ala.) diluted in PBS-T– BSA was added and incubated for 90 min at 37°C. After being washed with PBS-T, peroxidase-conjugated streptavidin (ICN Biomedical Inc.) was added and incubated for 1 h at 37°C. Finally, the plates were developed with orthopheylendiamine dihydrochloride (Sigma), the reaction was stopped after 15 min with 3 N H_2SO_4, and readings were taken at 490 nm. For the evaluation of specific anti-*S. pneumoniae* antibody response, plates were coated with 0.05 μg of recombinant pneumolysin/well and developed with biotinylated goat anti-mouse IgG as before. The His-tagged recombinant pneumolysin used was produced in *Escherichia coli* and purified by Ni^{2+} chelate affinity chromatography using a His-Trap kit (Amershan Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Assessment of total IgE in serum, as well as total IgA and IgE in BAL samples, was performed by ELISA, using a pair of specific rat monoclonal antibodies against each immunoglobulin (capture and detection) and standard preparations of mouse IgA or IgE for construction of the standard curves (all reagents were supplied by BD Pharmingen and were used according to the manufacturer's instructions).

Statistical analysis. Data are presented as the mean ± standard deviation of the mean. The two-tailed Mann-Whitney test or Student's *t* test was performed on the different sets of data. Differences were considered significant when the *P* value was <0.05.

RESULTS

Analysis of cell populations recruited to the lungs. Groups of mice were immunized with three doses of CBE or PBS (control group) administered 1 day apart. On days 1 and 7 after the third dose, the mice were sacrificed and the lungs were aseptically removed. Lung single-cell suspensions from five mice per group were stained using a DC marker combination: MHC class II plus CD11c (the αX chain of the p150,95 integrin abundant on murine DCs) and examined by FC. Pulmonary DCs were defined as a population of cells expressing high levels of MHC class II and CD11c (69). Figure 1 shows that 1 day after the last dose, mice immunized with CBE had higher numbers of pulmonary DCs than control mice ($n = 5$; $P < 0.05$; two-tailed Mann-Whitney test) and that this increment was still present 1 week later. The DCs obtained from CBE-immunized mice also showed increased expression of the activation markers CD80/CD86 ($P < 0.05$; two-tailed Mann-Whitney test) (Table 2).

Since a number of gram-negative bacteria were included in the extract, we evaluated the possible effect of LPS stimulation on the observed response. The endotoxin content in the BE, as well as in the colloidal formulation, was determined by means of the *Limulus* amebocyte lysate test as described in Materials and Methods. Groups of animals were immunized with CBE or PBS as before or received purified LPS (L6529; Sigma) with the same immunization scheme. Two different LPS control groups were included: one received an amount of LPS equivalent to that present in one i.n. dose of CBE (40 pg/mouse), and the other received a much larger dose (5 μg of LPS/mouse). DC recruitment to the lungs was assessed in all groups as before, and the results are summarized in Fig. 2. The results clearly showed that whereas CBE immunization induced a major influx of DCs into the lungs, LPS did not cause DC recruitment at either concentration evaluated (Fig. 2).

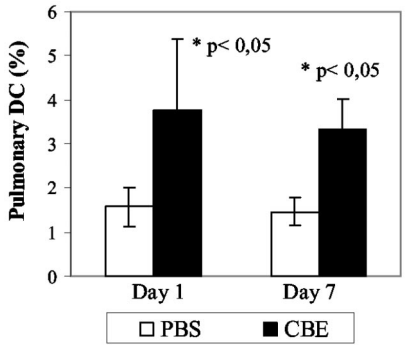

FIG. 1. Pulmonary DCs from immunized and control mice on days 1 and 7 after the last dose. The percentages of DCs in total lung cells were calculated by FC analysis of mice that received three i.n. doses of either CBE (immunized) or PBS (control) administered 1 day apart and were sacrificed 1 and 7 days later. For FC analysis, 10^6 cells from five mice per group were immunostained with Cychrome 5-CD11c and PE-MHC class II, and DCs were defined as described in Materials and Methods. The data were collected on a FACSCalibur. The results are expressed as mean ± standard deviation. *, $P < 0.05$ compared to the control group (two-tailed Mann-Whitney test), corresponding to five mice per group analyzed individually.

Analysis of May-Grunwald Giemsa-stained cytospin preparations showed a marked increase in the number of neutrophils, but not eosinophils, recruited to the lungs of mice receiving CBE compared with the PBS and LPS control groups (results not shown). Thus, the results show that the inflammatory process in the lungs was not the result of LPS stimulation.

The numbers of $CD4^+$ and $CD8^+$ T cells in the lungs from CBE-immunized and control mice were evaluated by FC on

TABLE 2. CD80 and CD86 mean fluorescence intensities of pulmonary DCs[a]

Activation marker	MFI[b]			
	Day 1		Day 7	
	Control	Immunized ($P < 0.05$[c])	Control	Immunized ($P < 0.05$[c])
CD80	47 ± 4	64 ± 13	49 ± 2	69 ± 8
CD86	47 ± 4	84 ± 18	71 ± 7	113 ± 9

[a] DCs were isolated from mice that received three consecutive doses of CBE (Immunized) or PBS (Control) and that were sacrificed 1 and 7 days later.

[b] Data represent mean fluorescence intensities (MFI) for five mice per group and per day of analysis ± standard deviations.

[c] $P < 0.05$ compared to control group (two-tailed Mann-Whitney test).

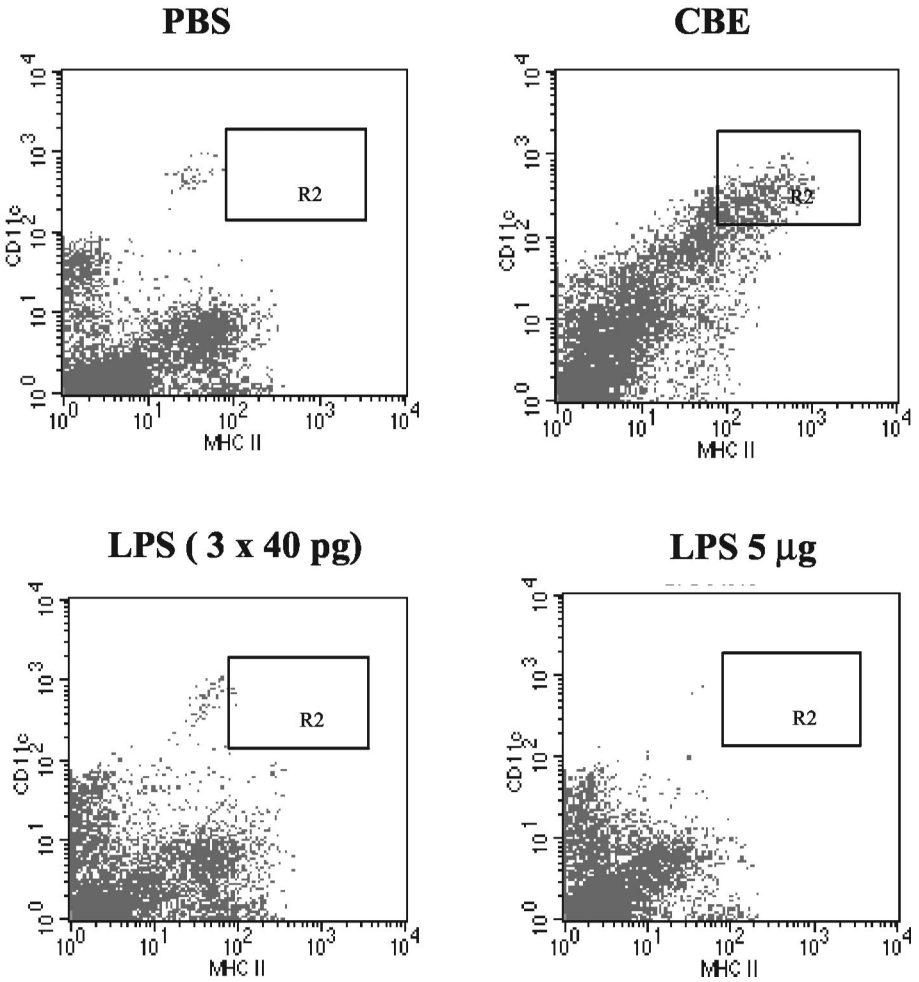

FIG. 2. Identification of pulmonary DC population. Lung single-cell suspensions prepared from mice that received three i.n. doses of CBE, PBS, or 40 pg of LPS administered 1 day apart or a single LPS dose of 5 μg and that were sacrificed 24 h later. Cells (10^6) from a pool of five mice per group were immunostained with Cychrome 5-CD11c, PE-MHC class II, and FITC-CD80 and -CD86. The data were collected on a FACSCalibur. DCs are represented in R2 ($CD11c^+$ MHC class II^+ cells).

day 7 after the last dose. No differences between the total T-cell numbers or the $CD4^+/CD8^+$ ratios in the two groups were found (24% ± 5% versus 25% ± 1% and 0.5 ± 0.1 versus 0.51 ± 0.08 for CBE immunized and PBS control mice, respectively).

Chemokine and proinflammatory-cytokine mRNA expression. Groups of mice were immunized as before and sacrificed either 4 or 24 h later. The lungs were harvested and used for RT-PCR analysis of whole-lung mRNA as described in Materials and Methods. Figure 3 shows the pattern of amplification for several chemokines and cytokines in lung homogenates. Immunization with CBE resulted in a clear increment over the baseline level of a number of CCL and CXCL chemokines, as well as in the expression of IL-1 and TNF-α. Notably, production of CCL3/MIP-1α, CCL4/MIP-1β, and the IFN-γ-inducible chemokines CXCL9/Mig and CXCL10/IP-10 was clearly augmented compared with control groups (Fig. 3). Additionally, the expression of CXCL1/KC and CXCL2/MIP-2, functional murine homologues for human IL-8 (78), was also augmented in CBE-immunized mice compared with control mice (Fig. 3).

Local cytokine production. First, we evaluated whether the immunization process would skew the cytokine environment in the lungs. We measured cytokine concentrations in BAL samples from the same mice sacrificed for FC analysis, as well as naïve mice. Levels of IL-10, IFN-γ, and IL-12 were evaluated by ELISA as described above. We found that BAL samples from naïve and control mice contained high concentrations of IL-10 and IL-12 and very low levels of IFN-γ and that these level were not significantly changed by the immunization procedure either on day 1 (Fig. 4) or day 7 (results not shown) after the last dose. The production of TNF-α upon immunization was also evaluated in BAL collected 1 day after the last dose. In this set of experiments, two LPS control groups were also included. Interestingly, whereas the CBE-immunized mice produced a modest but significant amount of TNF-α in the lungs, the LPS control mice that received an amount of LPS equivalent to that included in CBE (40 pg of LPS/mouse) did not produce TNF-α above the baseline level (Fig. 4D). This further supported the idea that the amount of LPS included in CBE cannot be solely responsible for the acute inflammatory

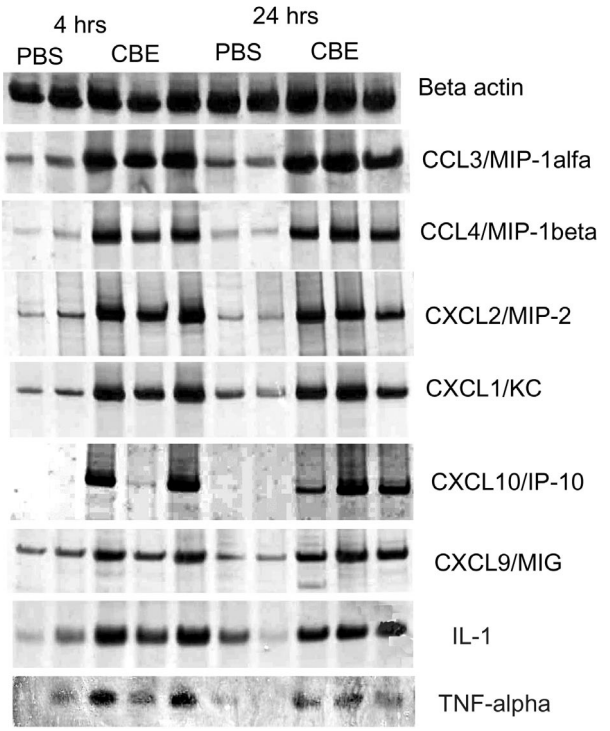

FIG. 3. Chemokine and proinflammatory-cytokine mRNA expression in lungs from mice that received three i.n. doses of CBE or PBS administered 1 day apart and were sacrificed 4 and 24 h later. Total lung RNA was extracted, and the expression of chemokines and cytokines was evaluated by RT-PCR. The amplification products were separated by electrophoresis in acrylamide gels and silver stained. The results are representative of three independent experiments.

response induced by the preparation. A positive control group for LPS-induced TNF-α production received a higher dose of LPS (5 μg/mouse). Mice from that group produced significant amounts of TNF-α in BAL samples (average, 600 pg/ml).

Alternatively, we assessed the recall immune response in the lungs after an antigenic challenge. Two groups of mice were immunized with CBE (by the same scheme used before), and 7 days later one of the groups received an additional i.n. dose of CBE (antigenic challenge) and the other served as a control. Both groups of mice were sacrificed 24 h later, and the lungs were harvested for RT-PCR analysis of IL-12 and IFN-γ mRNAs as described previously. Figure 5 shows that the in vivo restimulation with the antigen resulted in an increment in mRNA expression of both IL-12 and IFN-γ.

Serum antibody responses. For evaluation of antibody responses, groups of mice were immunized with three doses of CBE given on days 1, 25, and 39. A fourth dose was given on day 110 postpriming.

Figure 6 shows the IgG antibody response against bacterial components elicited in sera. Three i.n. doses were enough to elicit a specific antibody response, as detected by ELISA on day 54 postpriming, and those responses were significantly augmented by day 110 without further boosting ($P < 0.05$ compared to day 54; Student's t test; paired samples). A fourth dose given on day 110 did not further increase the response, as detected by ELISA of sera taken on day 120 (Fig. 6). Analysis of IgG1-IgG2a subclasses showed a balanced response among all sera, with high titers of both antibacterial IgG1 and IgG2a (Fig. 7). The antibody response was also evaluated against a defined antigen from *S. pneumoniae* as one of the organisms included in the extract. As can be seen, clear antibody responses against purified recombinant pneumolysin were elicited in all immunized mice (Fig. 6, inset).

An IgA antibody response against the BE was also detected in sera of immunized mice by day 54 ($P < 0.01$ compared with preimmunization sera; Student's t test) (Fig. 8). A specific antibacterial IgE response could not be detected at any time. However, the total serum IgE concentration showed a transient peak by day 54 ($P < 0.05$; Student's t test) that decreased later and remained at a low level (Fig. 9).

Local antibody production. Production of IgA was evaluated in the same BAL samples used for cytokine assessment. A significant increase in the concentration of total IgA was found as early as 7 days after immunization (results not shown). Furthermore, local production of IgA and IgE in the lungs was also evaluated by ELISA analysis of BAL samples taken 120 days after priming. The total IgA level was significantly increased in BAL samples from immunized mice compared to naïve mice ($P < 0.05$; Student's t test) (Fig. 10A), whereas the differences in IgE levels in BAL samples were not significant (Fig. 10B). Furthermore, significant levels of specific antibacterial IgA were detected in BAL samples from immunized mice, as assessed by two-tailed Mann-Whitney and Student's t tests (data not shown).

DISCUSSION

We conducted studies aimed at evaluating the i.n. route as an alternative for administration of BEs. A preparation consisting of small amounts of a BE formulated as a colloid was used for the studies. This preparation is presently being marketed in Uruguay as an immunostimulant for children suffering recurrent respiratory infections, with good performance in clinical practices. However, no clear characterization of the immunological effects had been conducted. In this work, we characterized the proinflammatory response induced in the lungs and assessed the development of specific immune responses against the bacteria included in the preparation.

We found that i.n. administration of such extracts resulted in an acute inflammatory response characterized by increased production of several chemokines and a rapid influx of DCs and neutrophils to the lungs. DCs are the principal resident antigen-presenting cell of the mouse lung (59, 65), and it has been shown that airway DCs are a very dynamic population that displays a high turnover rate, pointing to a central role for these cells in immune surveillance against invading pathogens through the airway mucosa (28, 29, 67). Once the DCs capture the antigen, induction of DC maturation occurs, and they migrate to local lymph nodes, where presentation to T cells takes place (1). Here, we show that i.n. immunization with inactivated bacteria resulted in the in vivo recruitment of large number of DCs to the lungs and that a high number of activated DCs can be found in the lungs up to 7 days after immunization (Table 2). This may suggest that the complex antigenic mixture administered had been captured and processed by these antigen-presenting cells and that the antigen-bearing

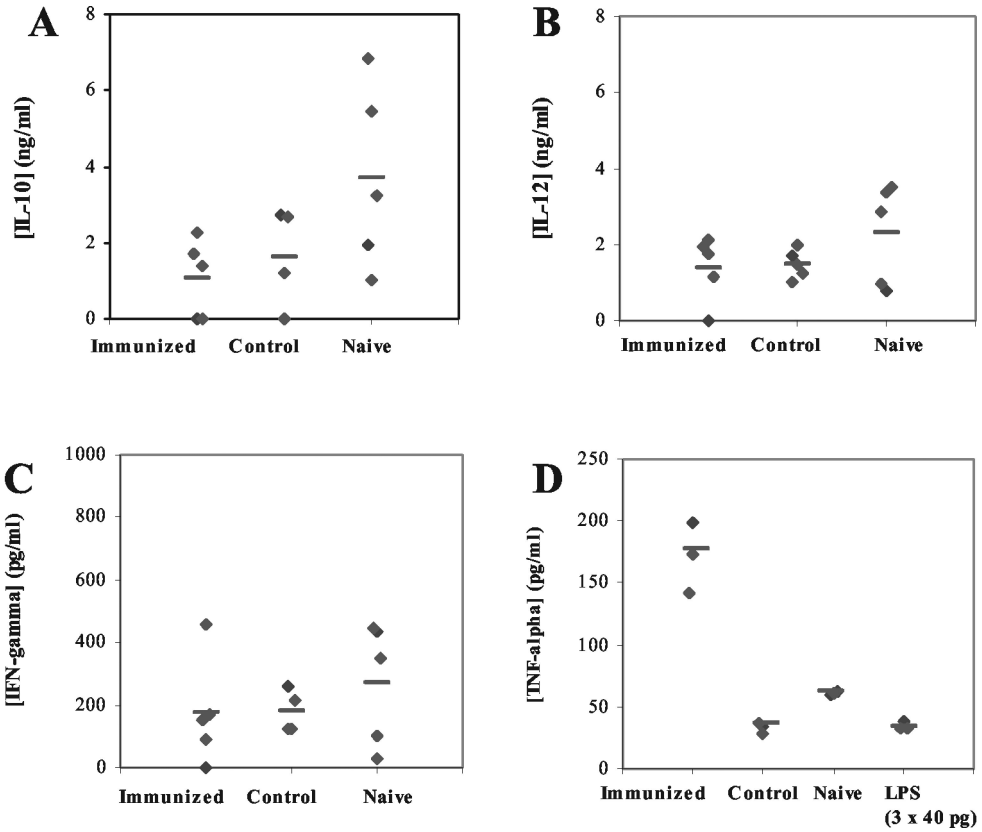

FIG. 4. Cytokine levels in BAL samples from naïve mice and mice that received three i.n. doses of CBE (Immunized) or PBS (Control) and were sacrificed 1 day later. IL-10 (A), IL-12 (B), and IFN-γ (C) concentrations were determined by ELISA. (D) For TNF-α, an additional group that received three i.n. doses of LPS (40 pg) was also analyzed. The results are expressed as the mean of duplicate optical-density values for individual mice and the mean values for the groups (horizontal bars).

DCs would eventually migrate to the local lymph nodes to initiate a specific immune response.

It is now clear that during an inflammatory response, both the chemokines produced and the receptors they use for signaling determine the extent, quality, and duration of the cellular infiltrate to the site of inflammation and also shape the adaptive immune response developed (58). A complex regulatory network of cytokine-chemokine interactions is emerging,

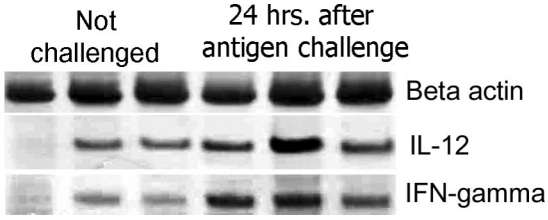

FIG. 5. Production of IL-12 and IFN-γ mRNAs in lungs after antigen challenge. Two groups of mice received three i.n. doses of CBE administered 1 day apart. Seven days later, one group received an additional dose of CBE, and the other group served as controls (Not challenged). All mice were sacrificed 24 h later, total lung RNA was extracted, and the mRNA expression of IL-12 and IFN-γ was evaluated by RT-PCR. The amplification products were separated by electrophoresis in acrylamide gels and silver stained. The results are representative of three independent experiments.

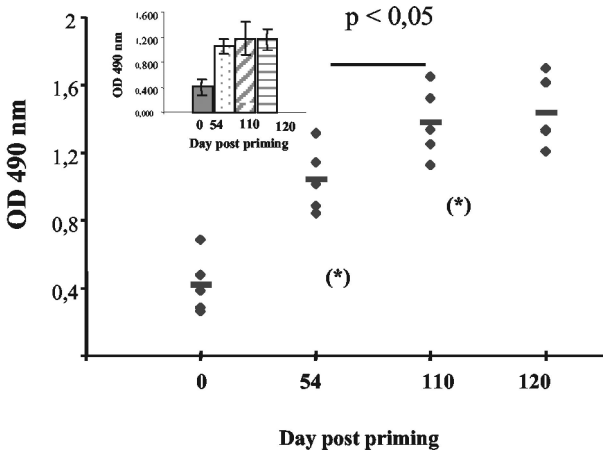

FIG. 6. Total IgG antibody response against CBE, as detected by ELISA analysis of sera taken on days 0, 54, 110, and 120 after priming. The results are expressed as the mean of duplicate optical-density (OD) values for individual mice immunized with CBE as described in Materials and Methods and the mean values for the groups of five mice (horizontal bars). *, $P < 0.05$ compared to day 0. (Inset) IgG antibody response against recombinant pneumolysin analyzed at the same time points. Data are the means for five mice per group ± standard deviation.

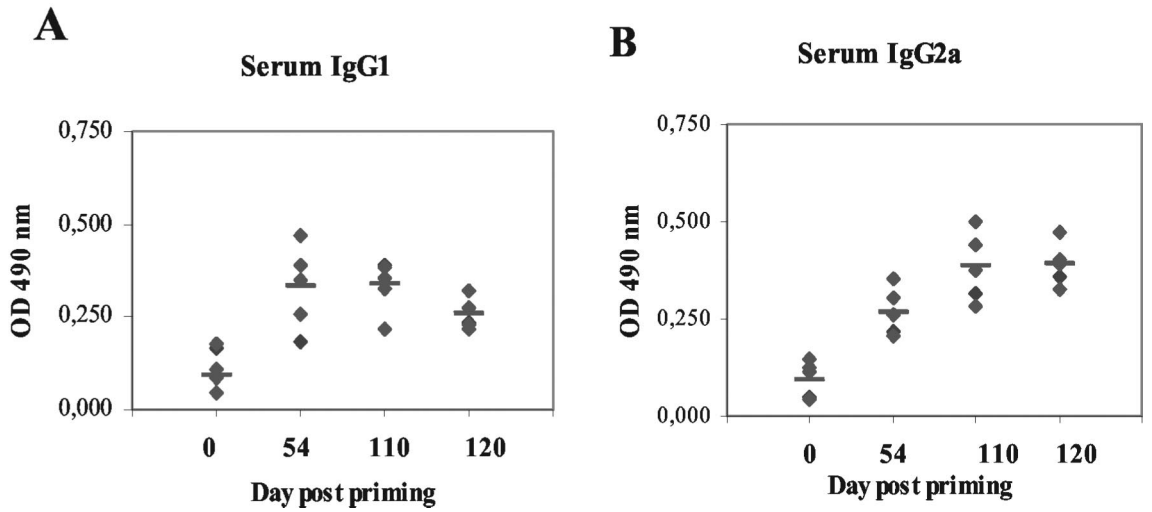

FIG. 7. Serum IgG1 (A) and IgG2a (B) against CBE, as detected by ELISA analysis of sera taken on days 0, 54, 110, and 120 after priming. The results are expressed as the mean of duplicate optical-density (OD) values for individual mice immunized with CBE as described in Materials and Methods and the mean values for the groups of five mice (horizontal bars).

but initial evidence indicates that this network may follow the basic rules of type 1-type 2 regulation (58). We observed increased production of CCL3/MIP-1α and CCL4/MIP-1β, which are considered to be involved in recruitment of immature DCs (37). Furthermore, we found increased production of IFN-γ-inducible chemokines: CXCL9/Mig and CXCL10/IP-10, and also CXCL1/KC and CXCL2/MIP-2.

We do not know which cells are producing these chemokines as a result of the immunization procedure, but it is interesting that we have found production of chemokines that involves CCR5 (MIP-1α and MIP-1β) and CXCR3 (IP-10 and MIG) signaling (56, 77). These two receptors have been shown to be involved in shaping type 1 responses (36, 37), and it has been proposed that CXCR3 ligands (IP-10 and MIG) are important components of the trafficking and recruitment of Th1 cells (22, 46, 57). Overall, these results suggest that this immunization protocol could induce a local immune response with a strong type 1 component. Overexpression of IP-10 in the lungs has also been related to increased airway hyperreactivity, if associated with eosinophil and T-cell recruitment (46). We observed a dramatic change in the production of IP-10 as a result of the immunization procedure (Fig. 3). These results were also confirmed by real-time PCR (results not shown). However, eosinophil recruitment was not observed, and the number of $CD4^+$ $CD8^+$ T cells in the lungs was not different from that in naïve mice, suggesting that increased airway hyperreactivity may not be a feature of this system.

On the other hand, we observed that BAL samples of naïve mice or mice that received PBS had high concentrations of IL-10 and IL-12 and low concentrations of IFN-γ and that these levels were not changed by immunization with CBE (Fig.

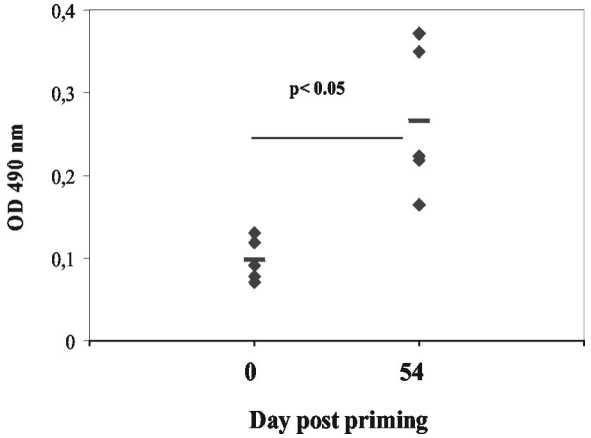

FIG. 8. IgA response against CBE as detected by ELISA analysis of sera taken on days 0 and 54 after priming. The results are expressed as the mean of duplicate optical-density (OD) values for individual mice immunized with CBE as described in Materials and Methods and the mean values for the groups of five mice (horizontal bars). $P < 0.05$ compared to day 0 (two-tailed Student t test).

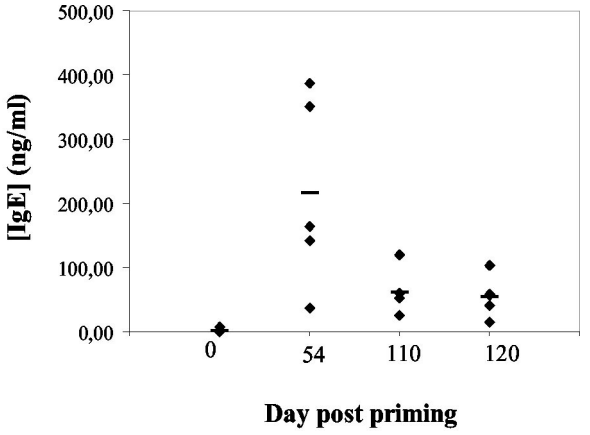

FIG. 9. Total IgE antibody concentration in sera taken on days 0, 54, 110, and 120 after priming from mice immunized with CBE. Each value is the average for duplicate ELISA determinations for individual mice, and the horizontal bars represent the mean values for the groups.

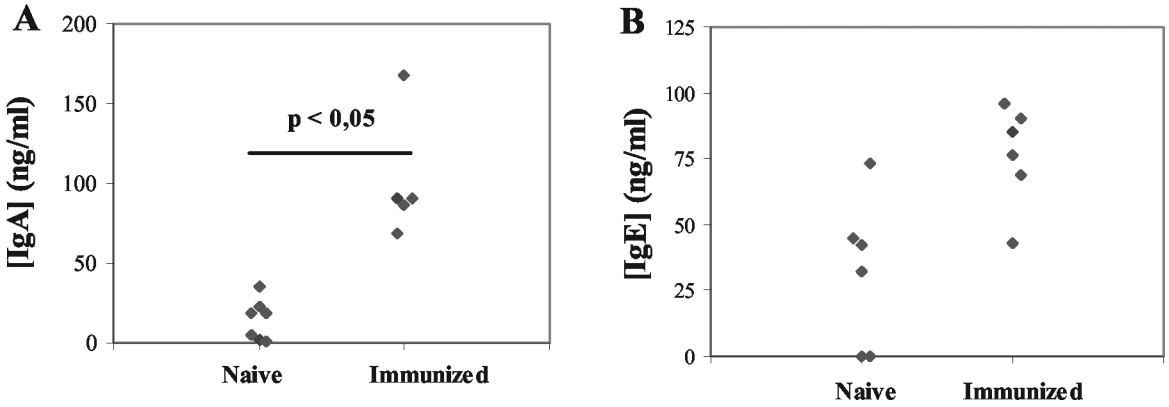

FIG. 10. Total IgA and IgE antibody concentrations in BAL samples from naïve and immunized mice 2 weeks after the last booster (day 120 after priming). The results are expressed as mean concentrations fromduplicate ELISA determinations for individual mice.

4). It has been shown in vitro that immature DCs from the respiratory tract constitutively express IL-10 and that this expression is shut down upon activation induced by preculture of the cells with granulocyte-macrophage colony-stimulating factor (65). We observed high in vivo IL-10 expression in BAL samples from naïve or PBS-immunized mice, but we did not find a clear reduction in the IL-10 concentration in BAL samples upon activation of DCs. It is possible that the observed effect was the consequence of the rapid turnover of these cells (59, 65–67), since a continuous influx of immature DCs and migration of the activated cells to regional lymph nodes would act to maintain a high proportion of IL-10-producing cells within the lungs. Also, it could be that we were detecting preformed IL-10, and the possibility that analysis of cytokine mRNA production would demonstrate a neat reduction in IL-10 expression after immunization cannot be ruled out. However, we consider that there is another possible explanation for these findings. Different subsets of DCs that have the capacity to induce preferential priming for either Th1 or Th2 cells (11, 21, 29, 39, 53) have been identified in mice and humans, and it has been proposed that IL-10 production is a potential marker for one of them (29). It could be that this immunization procedure also activates the subset favoring Th2, pointing to a more balanced Th1-Th2 environment within the lungs. This would be in line with our findings of balanced IgG1 and IgG2a antibody responses against the bacteria included in the preparation (Fig. 7).

Analysis of the antibody response showed that 3 i.n. doses of the BE were enough to elicit long-lasting IgG and IgA responses (Fig. 6 and 8). Interestingly, the specificity of the response could also be observed by assessing the production of antibodies directed to a defined bacterial antigen from one of the organisms included (*S. pneumoniae*). The increase in bacterium-specific IgG antibodies after the boosters (Fig. 6) suggests the induction of immunological memory against the bacteria. To our knowledge, this is the first report showing that BEs can elicit a specific immune response after so few doses. Moreover, the observed increased production of IFN-γ and IL-12 in the recall response after antigenic challenge (Fig. 5) could suggest that specific T cells are being activated by the immunization.

The total IgA concentration in BAL fluid was significantly increased in immunized mice (Fig. 10). Previous work with orally administered BEs has similarly shown an increased concentration of total IgA in saliva (10, 49), suggesting that BEs stimulate mucosal immune mechanisms that lead to the production of local secretory IgA (sIgA). In addition, we detected the production of specific anti-bacterial sIgA in BAL samples. A major role of local sIgA in mucosal defense against invading pathogens has long been clearly demonstrated (42, 74), and our results can be taken to suggest that i.n. administration of BEs may result in specific immune protection of those sites.

We found a peak of total IgE concentration in serum 2 weeks after the third dose (day 54) that decreased later (day 110) and did not increase after the fourth dose (Fig. 9). A rise in the level of specific antibacterial IgE that could explain the increase in total IgE production by day 54 could not be demonstrated, although that may be due to the fact that a low IgE/IgG ratio in serum could result in low sensitivity of the ELISA. An initial increase in the total serum IgE concentration would be consistent with the described Th2-like initial response to inhaled antigens (29, 30, 43, 60, 61). However, the later decrease in the IgE level may suggest that the BEs exert an immunomodulatory effect on IgE production. On the other hand, no significant differences were found in total IgE concentrations in BAL samples on day 120 (Fig. 10B). Although we cannot exclude the possibility that such differences could be present earlier (on day 54, as in serum), the lack of differences on day 120 is particularly relevant, and together with the lack of eosinophil recruitment to the lungs, it suggests that i.n. administration of these preparations does not result in a chronic augmentation of IgE in the airways, which would be a serious drawback considering the role of lung IgE in the pathogenesis of atopic disorders (72).

The two main differences between our work and previous related work with BEs are the use of a colloidal formulation for the extract and the i.n. route for delivery. The superior immunogenicity of a microparticle vaccine system compared with soluble antigens for i.n. immunization is still a matter of debate, with some authors arguing that the former results in stronger antibody responses whereas others found a decreased or unchanged response compared with i.n. administration of the antigen in a soluble form (19). However, in a preliminary study aimed at comparing CBE with a similar noncolloidal

preparation, we observed that the colloidal preparation was more immunogenic than an identical noncolloidal extract when administered by the i.n. route (our unpublished results).

There are several works showing that i.n. administration of whole-cell inactivated bacteria can induce effective immunity in the absence of adjuvants (17, 27, 32, 34). In particular, the administration of whole-cell pertussis vaccine by the i.n. route has proven to produce antigen-specific T-cell responses in human volunteers (5) and strong systemic and mucosal antibody responses in goats (6). Moreover, it has also been demonstrated that mice immunized i.n. with whole-cell heat-inactivated *S. pneumoniae* are protected against a challenge with live pneumococci (32). Taken together, these reports suggest that the i.n. route might be an improvement for the administration of inactivated bacteria. Our results are in agreement with those findings, and we extend them here by showing that i.n. immunization with small amounts of a BE formulated as a colloid can be a simple alternative to elicit specific immune responses against the different bacteria included in the preparation.

In summary, the results presented here demonstrate that in addition to their classic effect as nonspecific immunostimulators, BEs formulated as a colloid and delivered i.n. can induce specific antibacterial antibody responses in sera and in the airway mucosa. By combining the use of such preparations with new tools to potentiate and particularly to modulate local immune responses, such as molecular adjuvants and codelivery of cytokines, it could be possible to define new cost-effective immunotherapies for the prevention and treatment of respiratory infections and otitis media. Work with that goal is under way.

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