

Immune Response in the Lungs following Oral Immunization with Bacterial Lysates of Respiratory Pathogens

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We have investigated the local immune response of the BALB/c mouse respiratory tract after oral immunization with a bacterial lysate of seven common respiratory pathogens. After two immunizations on five consecutive days, we examined the immunoglobulin (immunoglobulin G [IgG], IgM, and IgA) secretion rates of cells isolated from the lungs and compared them with those of spleen cells of orally immunized and nonimmunized animals by using a new test system based on time-resolved fluorescence. The procedure followed the principle of the classical ELISPOT test with nitrocellulose-bottomed microtiter plates, but europium (Eu³⁺)-linked streptavidin rather than enzyme-conjugated streptavidin was used, with the advantage of quantifying secreted immunoglobulins instead of detecting single antibody-secreting cells. Lymphocytes isolated from the lungs of treated animals revealed significant increases in total and antigen-specific IgA synthesis compared with the rates of the controls, whereas IgG and IgM production rates showed no remarkable differences. In addition, the sera of treated mice revealed higher antigen-specific IgA titers but not increased IgM and IgG levels. We conclude that priming the gut-associated lymphoid tissue with bacterial antigens of pneumotropic microorganisms can elicit an enhanced IgA response in a distant mucosal effector site, such as the respiratory tract, according to the concept of a common mucosa-associated immune system.

Mucosal surfaces, including those of the gastrointestinal tract, airways, the oral cavity, and the urogenital tract, represent the first barrier of local defense against invading bacterial, viral, and parasitic pathogens (8).

Oral applications of antigens have been shown to induce dissemination of lymphoid cells from the gut-associated lymphoid tissue to distant mucosa-associated effector sites, such as the respiratory tract, the genitourinary tract, and various excretory glands, where these cells proliferate and differentiate into effector cells (17, 26). The increased production of antigen-specific secretory immunoglobulin A (IgA) observed in remote secretions, including saliva, tears, and fluids from bronchial and gastrointestinal washes (3, 5, 7, 14, 27), results in local immune protection.

This communication between different mucosal tissues forms the basis of oral immunization strategies (15). Whereas parenterally applied vaccines do not induce secretory antibody titers at mucosal surfaces (11), oral and intranasal immunizations can effectively induce antigen-specific secretory IgA secretion at the application site (1, 4).

In recent years, several investigators have reported the development of oral vaccination strategies that elicit protective secretory IgA antibody responses on mucosal surfaces; these responses are induced by replicating as well as nonreplicating agents. Moreover, many attempts to improve antigen delivery to mucosal lymphoid tissues, have been made, e.g., the use of cholera toxin B subunit (33), microparticles (24, 25), immunostimulating complexes (20), and liposomes (13).

We investigated the immunomodulatory and protective effects of a bacterial lysate of seven common respiratory patho-

gens on the respiratory tract, where bacterial infections frequently begin. Because of the clinical relevance of respiratory infections and the importance of the development of new immunization strategies, we examined whether this bacterial lysate could improve the local immune response in the respiratory tract following initial immunization at a remote site, namely, the intestinal tract.

Our previous studies demonstrated enhanced peripheral polymorphonuclear leukocyte functions after oral application of bacterial antigens, as reflected by a significantly increased oxidative burst activity (10). Van Daal et al. reported a reduction of intrapulmonary inflammatory reactions to infection with *Streptococcus pneumoniae* and an enhancement of pulmonary defense mechanisms mediated by increased gamma interferon production (32). Furthermore, we demonstrated that the orally applied bacterial lysate appreciably enhanced the rates of migration of intestinal lamina propria lymphocytes and Peyer's patch lymphocytes to the lungs when injected intravenously into untreated syngeneic recipients (28).

In the present study, we examined induction of Ig (IgG, IgM, and IgA) responses in the respiratory tracts, spleens, and sera of mice immunized orally with bacterial lysates of pneumotropic microorganisms. To evaluate the rates of antibody synthesis of lung lymphocytes and splenocytes, we developed a new test system based on a time-resolved fluoroimmunoassay (TR-FIA) (29). The procedure described here follows the principle of the classical ELISPOT test with nitrocellulose-bottomed microtiter plates (19), but we employed Eu³⁺-linked streptavidin rather than enzyme-labelled streptavidin. Secreted substances bound to the solid phase were detected by fluorescence measurement rather than by microscopic visualization and counting. With an appropriate enhancement solution, Eu³⁺ cations can be dissociated from the nonfluorescent streptavidin-Eu³⁺ chelate bound to the solid phase in solution. After dissociation, these freed cations instantly form new, highly fluorescent chelates with components of the enhance-

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ment solution that can then be quantified by fluorescence measurement (16).

MATERIALS AND METHODS

Animals and immunization procedure. Inbred 5- to 7-week-old female BALB/c mice were obtained from the Research Institute for Laboratory Animal Breeding, Himberg, Austria, and maintained at the Central Laboratory Animal Facilities of the Medical School, University of Innsbruck, Innsbruck, Austria.

The bacterial lysate (LW50020; kindly provided by M. Ellenrieder, Luitpold-Pharma GmbH, Munich, Federal Republic of Germany) contained at least 10^{10} (per gram) of each of the following heat-inactivated, mechanically disintegrated, lyophilized bacteria: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Branhamella catarrhalis*, and *Klebsiella pneumoniae*. The bacterial lysate was mixed at a ratio of 3:100 with mannitol and dissolved in phosphate-buffered saline (PBS), pH 7.2.

According to the previously established schedule (28), the mice were immunized orally by gavage for five consecutive days with 15 mg of bacterial lysate per kg of body weight in a total volume of 200 μ l of PBS. After 12 days, the immunizations were repeated for another 5 days. One week later, the animals were sacrificed and organs were removed for cell isolation.

Isolation of spleen and lung cells. Splenocytes were isolated as described previously (28). Intraparenchymal lung lymphoid cells were isolated by a procedure modified by us and originally described by Abraham et al. (2). Briefly, cells were isolated and pooled from 10 mice in each group. After the lung vascular bed was flushed by injecting 4°C Hanks balanced salt solution (Sigma) into the right ventricle, the lungs were excised, avoiding the peritracheal lymph nodes. After being washed in Hanks balanced salt solution, the lungs were minced and digested twice for 1 h at 37°C in 40 ml of RPMI 1640 (Biochrom KG, Berlin, Federal Republic of Germany) containing 10% newborn bovine serum (HyClone Sterile Systems, Inc., Logan, Utah), 100 U of penicillin and 100 μ g of streptomycin (Biological Industries, Beth Haemek, Israel) per ml, 1 mg of DNase (Sigma Chemicals) per ml, and 10 U of purified collagenase from *Clostridium histolyticum* (CLSPA; Worthington Biochemical Corp., Freehold, N.J.) per ml in a shaking water bath (200 rpm). The minced pieces were then forced through 200-mesh stainless steel sieves, and the resultant cell suspensions were filtered through a glass bead-filled syringe to eliminate debris and cell aggregates. For further purification, the isolated lung lymphoid cells were centrifuged over a 40 to 80% Percoll gradient (Pharmacia Fine Chemical, Uppsala, Sweden) for 20 min at 600 \times g. Cells were recovered from the interface, washed twice with RPMI 1640 supplemented with 10% newborn bovine serum, and counted by trypan blue exclusion (viability, >90%).

Detection of total- and specific-antibody-secreting cells by TR-FIA (29). Nitrocellulose-bottomed microtiter plates (Multiscreen-HA; Millipore Corp., Bedford, Mass.) were coated overnight at 4°C either with 2 μ g of purified goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, Ala.) per ml or with 2.5 μ g of bacterial lysate per ml and, for control purposes, with PBS (100 μ l per well). To remove unadsorbed proteins, the plates were washed three times with PBS and free binding sites were blocked with 200 μ l of 1% bovine serum albumin (BSA) (Sigma)-PBS for 1 h at room temperature.

Cells isolated from immunized and nonimmunized mice

were resuspended in RPMI 1640 supplemented with 10% newborn bovine serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml and were added to individual wells in appropriate numbers (10^6 cells per well). The cells were then cultured at 37°C in an incubator with 5% CO₂ and 100% humidity for 4 h. The plates were washed thoroughly with PBS-0.05% Tween 20 and incubated overnight at 4°C with 100 μ l each of biotinylated affinity-purified goat anti-mouse μ -, γ -, and α -chain-specific antibodies (Southern Biotechnology Associates) (diluted 1:1,000 in 1% BSA-PBS). On the following day, the plates were rinsed five times with PBS-0.05% Tween 20 and incubated for 1 h at room temperature with 100 μ l of Eu³⁺-labelled streptavidin per well (diluted 1:1,000 in assay buffer containing 0.1 M Tris-HCl, 0.15 M NaCl, 0.05% NaN₃, 20 mM diethylene triaminepenta-acetic acid, 0.5% BSA, 0.05% bovine gamma globulin, 0.01% Tween 20 [pH 7.55]; Kabi Pharmacia, Uppsala, Sweden). Unbound streptavidin was removed by washing the plates five times with PBS-Tween 20 prior to the addition of 150 μ l of enhancement solution (15 μ M β -naphthoyltrifluoroacetone, 50 μ M tri(*n*-octyl)phosphine oxide, 6.8 mM potassium hydrogen phthalate, 100 mM acetic acid, 0.1% Triton X-100 [pH 3.2]; Kabi Pharmacia), which efficiently releases Eu³⁺ from the streptavidin. After 15 min of incubation on a plate shaker, 100 μ l from the total volume was transferred with a multichannel pipette to a 96-well polystyrene plate (Immuno Module, Maxisorp C12; Nunc, Roskilde, Denmark). Finally, the fluorescence was measured for 1 s in a time-resolved fluorometer (Arcus 1230; Wallac Oy, Turku, Finland) at an excitation wavelength of 340 nm and with an emission of 614 nm.

Detection of total and specific serum antibodies. The levels of total and specific serum antibodies (IgA, IgG, and IgM) were quantified by TR-FIA. Mice were bled from the retro-orbital plexus, the blood samples were centrifuged for 5 min at 15,000 \times g, and the sera were collected and stored at -70°C until analysis. Serum dilutions (1:1,000 for total Ig and 1:10 for antigen-specific Ig) were applied to nitrocellulose-bottomed microtiter plates (Multiscreen-HA; Millipore) coated with purified goat anti-mouse Ig for total Ig and with bacterial lysate for antigen-specific Ig, as described above, and incubated for 2 h at room temperature. Biotinylated goat antibodies directed against mouse IgA, IgG, and IgM were used as secondary antibodies and were detected with Eu³⁺-labelled streptavidin.

Statistical evaluation. In the experiments examining total and antigen-specific Ig secretion of treated and control animals (each group consisting of 10 mice), lung lymphocytes and splenocytes were isolated and pooled for further analysis. The fluorescence intensity was measured in triplicate, and the data are the means + the standard errors of the means (SEM), as indicated in the figure legends. Comparisons of group means were performed by the unpaired Student *t* test. A *P* value of less than 0.05 was considered to be significant.

RESULTS

We used our modified TR-FIA to examine whether repeated oral immunizations with pneumotropic bacterial lysates can stimulate an Ig response in the respiratory tract. Cells producing total Ig and those producing antigen-specific IgG, IgM, and IgA were isolated from the lungs and (for comparison) from the spleens of mice immunized orally with the bacterial lysate LW50020 and were assessed. After two immunizations, the animals were sacrificed and cells from the tissues to be investigated were isolated and maintained under cell culture conditions for 4 h in nitrocellulose-bottomed microtiter wells coated either with a capture goat anti-mouse Ig, for

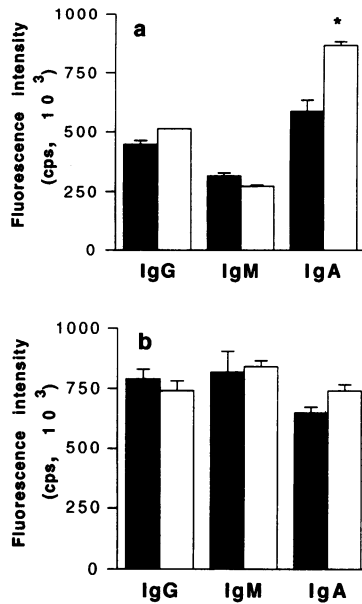


FIG. 1. Ig isotype (total IgG, IgM, and IgA) secretion in the lungs (a) and spleens (b) of mice. Mice received 15 mg of either mannitol (controls [■]) or LW50020 (□) per kg of body weight. The mice were orally immunized for five consecutive days. After 12 days, the immunization was repeated for another 5 days, and 1 week thereafter, the animals were sacrificed and the lungs and spleens were removed. Ten mice from each group were used, and isolated cells were pooled for further analysis. Data are means + SEM from triplicate wells. The asterisk indicates a value significantly different from that of the control ($P < 0.05$). The results are representative of three separate experiments.

detection of total Igs, or with the bacterial antigens, for detection of antigen-specific Igs. The procedure described here follows the principle of the classical ELISPOT test, but secreted substances bound to the solid phase were detected by overall fluorescence measuring of Eu^{3+} rather than by microscopic spot counting.

As shown in Fig. 1a and 2a, the orally applied bacterial lysate effectively induced a marked IgA response in the lungs. In fact, 12 days after the second immunization period, the IgA secretion of isolated lung lymphocytes was enhanced in comparison with that of cells isolated from the untreated animals. Oral immunization with LW50020 resulted in significantly ($P < 0.05$) increased production of antigen-specific IgA (33,600 cps) as well as an increase in the total pulmonary IgA response (865,000 cps) compared with the levels of untreated controls (18,600 and 585,000 cps, respectively). No significant alterations in IgG (512,000 cps) or IgM (273,000 cps) synthesis of cells isolated from treated mice were found in comparison with the levels of their nonimmunized counterparts (447,000 and 316,000 cps, respectively).

As shown in Fig. 1b and 2b, oral immunization with the bacterial lysate elicited no significant stimulation of antibody response in the spleen.

To determine whether these bacterial antigens could enhance the antibody response in peripheral blood of orally immunized animals, we measured the total and antigen-specific antibodies (IgG, IgM, and IgA) of treated and untreated mice by TR-FIA. A small, but significant, increase (20%; $P < 0.05$) of antigen-specific IgA was found after two immunization periods. No differences in specific IgG and IgM were observed. Similarly, the total serum IgG, IgM, and IgA levels of the

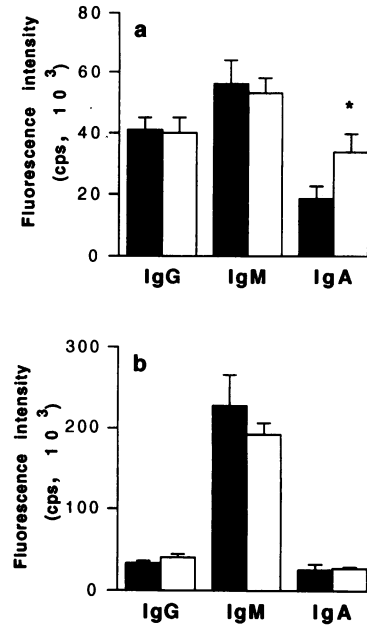


FIG. 2. Induction of antigen-specific Ig secretion (IgG, IgM, and IgA) after oral immunization with the bacterial lysate in the lungs (a) and spleen (b). Mice received 15 mg of either mannitol (controls [■]) or LW50020 (□) per kg of body weight. The animals were immunized as described in the legend to Fig. 3. Data are means + SEM from triplicate wells. The asterisk indicates a value significantly different from that of the control ($P < 0.05$). The results are representative of three separate experiments.

immunized group were not significantly affected in comparison with the levels of the untreated controls (Fig. 3).

DISCUSSION

The notion of a common mucosal immune system forms the basis for oral immunizations against infections arising in distant effector sites, such as the respiratory and urogenital tracts, or against dental caries (12, 17, 23).

Because of the importance of developing new vaccination strategies against respiratory infections, we were interested in searching for a local immune response in the respiratory tract by oral priming of the gut-associated lymphoid tissue with bacterial lysates of seven microorganisms commonly associated with respiratory infections, such as sinusitis and bronchitis.

The efficacy of orally administered antigens for induction of local immunity has been extensively examined by indirect determinations of specific antibodies or cytokine levels in biological fluids, such as saliva, bronchial lavages, gastrointestinal washes, or colostrum (18). However, these indirect measurements do not exactly reflect the local immune response because of the well-known individual variability and very high standard deviations of these parameters. In fact, these molecules are produced transiently and in very small quantities, have short half-lives, and are protease sensitive. Moreover, the anatomical localization of secretion cannot be exactly identified.

To avoid this problem, we addressed the humoral response on the cellular level in our experiments, evaluating the rates of antibody synthesis of lymphocytes obtained from different lymphoid tissues. Using the classical ELISPOT test, several authors have shown that oral or intranasal immunization with a variety of antigens combined with adjuvants, such as lipo-

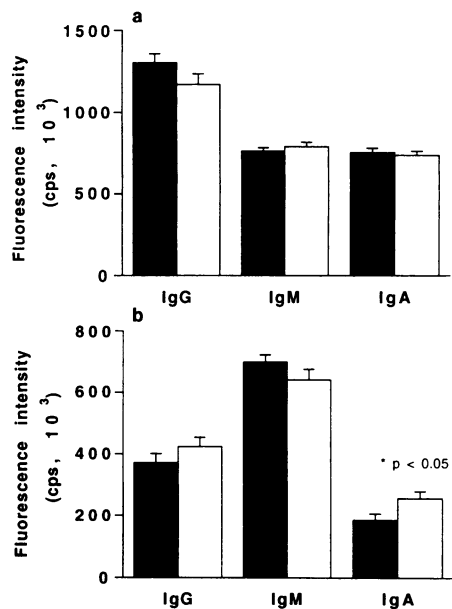


FIG. 3. Total (a) and antigen-specific (b) Ig (IgG, IgM, and IgA) response in the sera of control mice and mice orally immunized with 15 mg of immunomodulator per kg of body weight. Each group consisted of 18 animals, and the results are means \pm SEM for serum samples diluted 1:1,000 (total Ig) and 1:10 (antigen-specific Ig). Antigen-specific IgA titers for mice orally immunized were significantly greater ($P < 0.05$) than those for control animals.

somes and the cholera toxin B subunit (1, 22, 33), could increase the number of antibody-secreting cells at the application site.

The method we used is based on TR-FIA, which employs Eu^{3+} -linked streptavidin to quantify the Ig production rate of cells in the lungs and spleen. A previous methodological study showed that this new approach is very sensitive and specific and can be used to assess the anatomic localization of antibody-secreting cells and to quantify cell-secreted products. Synthesized Igs bound to the solid phase on a nitrocellulose-bottomed plate were detected by fluorescence measurement rather than microscopic visualization and counting. In a previous article (29), we described the precise quantification of secreted products from cell suspensions, expressed in fluorescence intensity (counts per second), and the good correlation with the original ELISPOT technique (30).

The amount of Ig isotype production was characteristic for each isolated cell type. During the 4-h culture, these cells showed the capacity to produce all three isotypes, with a slight predominance of IgA, as documented by others (2). In contrast, splenocytes contained cells capable of secreting all three isotypes equally.

Regarding the Ig production rates (IgG, IgM, and IgA) in the respiratory tracts of orally immunized animals, our data support the concept of a common mucosal immune system. The main finding of the study was that commercially available LW50020 was effective in inducing a significant IgA antibody response in the murine respiratory tract. In fact, an increased secretion of total IgA as well as antigen-specific IgA was measured after two immunization periods with orally applied killed respiratory pathogens compared with that of untreated controls. In contrast, no significant induction of Ig production could be detected in the spleen.

The observation that antigen-specific IgA levels in the sera of treated animals are increased in comparison with those of untreated ones could point to an early systemic immune response after oral immunization.

To clarify the mechanisms involved in mucosal immunity, it is of primary interest to define the cell type involved in the generation of the immune response in the respiratory tract. As is already well known, one possibility is that antigen-stimulated IgA lymphoblasts migrate via the lymphatics from the gut-associated lymphoid tissue into the blood, localize in mucosal tissues, and there secrete IgA (18). Migration of T lymphocytes from inducer sites to other not-directly-stimulated mucosal surfaces can also be envisaged (23). It is well known that T cells induce a marked increase in IgA production through secretion of different cytokines (6). Cytokines such as interleukin-4 (IL-4) and IL-5 or growth factors such as transforming growth factor β can evoke B-cell differentiation, maturation, and a heavy-chain isotype switch from IgM and IgG to IgA (9, 21). Taguchi et al. (31) showed that in IgA effector sites, such as the intestinal lamina propria, a greater number of IL-5-producing Th_2 -type cells was observed than gamma interferon-secreting Th_1 cells. In contrast, equal numbers of Th_1 - and Th_2 -type cells were detected in Peyer's patch CD4^+ T-cell fractions. The frequency of Th_1 and Th_2 cells in the inducer and effector sites of the mucosal immune system may determine the local isotype-specific antibody response. Furthermore, exogenous antigens may thus also contribute to induction of these different cell clones at these sites.

Supported by these observations, our current studies are investigating which cell type(s) (Th_1 - and/or Th_2 -type CD4^+ T cells) is involved in the observed immune response in the respiratory tracts of immunized animals. In addition, we will examine whether cytokine release (IL-2, -4, -5, -6, and -10; gamma interferon; and tumor necrosis factor alpha) can be modulated or stimulated in the respiratory tracts of mice immunized by the oral route. The sensitive TR-FIA technique described herein for detection of antibody-secreting cells has been optimized for the detection of a variety of other cell-secreted substances, such as cytokines.

In conclusion, the results indicate that priming of the gut-associated lymphoid tissue with bacterial lysates of respiratory pathogens enhances IgA production in the lungs. This correlates well with our previous findings that oral immunization leads to an increased appearance of lamina propria lymphocytes and Peyer's patch lymphocytes in the lungs after intravenous injection into syngeneic recipients (28) and also provides a rational basis for explaining the clinically observed effects of this type of immunomodulation.

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