Appearance of Specific Antibody-Bearing Cells in Human Bronchial Mucosa after Local Immunization with Bacterial Vaccine

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The immune response to local in vivo inhalation of a lysed bacteria vaccine was assessed in surgical specimens of main-stem bronchi from patients who had undergone pneumectomy for cancer. The patient population included 22 subjects; 11 of these received the aerosol vaccine twice a day for 10 days prior to surgery, while the remaining 11 patients were used as controls and were not immunized. The submucous glands of immunized subjects showed significantly more cells than did those of the controls, i.e., 62 ± 8 versus 37 ± 7 , respectively ($P < 0.05$). The following five antigens were chosen for study by fluorescence assay: *Streptococcus pneumoniae* types II and III, *Haemophilus influenzae*, *Streptococcus* sp. strain D19, and *Klebsiella pneumoniae*. An immunization-dependent correlation was found between immunoglobulin A, immunoglobulin A-bearing cells, and specific antibody-bearing cells on the one hand and three of the five antigens (*S. pneumoniae* types II and III and *Streptococcus* sp. strain D19) on the other hand. This is the first time that a relationship has been established between bacterial immunization of the lower respiratory tract and local immunoglobulin production in humans.

Antibody responses are a crucial part of the immunologic reactivity of the lung membrane against inhaled antigens (2, 8, 13, 17, 19, 25). The appearance in the lung of antibody-bearing cells (ABC) in response to immunization or infection has been demonstrated in a variety of species (2, 13, 25). As yet, experiments aimed at enhancing antibody-mediated bactericidal activity have not yielded conclusive results. Regarding these experiments, the Food and Drug Administration concluded that "the weight of clinical evidence supporting the effectiveness of bacterial vaccines in humans is largely uncontrolled and anecdotal but it comprises many thousands of patients and its use spans a period of at least 40 years" (19). Interest in bacterial vaccines has, however, been revived by the fact that vaccination with pneumococcal polysaccharide can provide effective protection against pneumococcal pneumonia and bacteremia in adults (1). Subsequent studies of this finding have emphasized the role of opsonins in promoting phagocytosis (11).

Most investigative research into the immunologic responses of bronchial mucosa to local bacterial vaccination have been carried out on nasal secretions from the upper respiratory tract (5, 35) or on alveolar lung fluid (2, 4). Little attention has been given to responses in the trachea and main-stem bronchi, mainly because of the difficulty in obtaining tissue samples. For technical reasons that will be explained below, fiberoptic biopsies do not provide adequate experimental material. This lack of material has been a major obstacle to the extensive study of aerosol immunization, which has been shown to be more effective than the systemic route in enhancing the defense mechanisms of the respiratory membrane in animals (14, 16, 22, 33).

The purpose of the present in vivo study was to determine

whether bacterial aerosol challenge enhanced immune responses and, in particular, the production of ABC in human bronchi. Since no monovalent bacterial antigen is available for aerosol immunization in humans, we used a bacterial extract that has been prescribed as a vaccine in Europe for over 10 years. To our knowledge, this study is the first time that aerosol bacterial challenge and ABC counts have been correlated in human bronchi. Thus, besides giving positive evidence for enhancement of immune responses, this study provides a model for investigating immune responses to local immunization.

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MATERIALS AND METHODS

Patients. Bronchial tissue was obtained from 22 patients who had undergone pneumectomy for histologically confirmed carcinoma (in 19 patients) or bronchiectasis (in 3 patients). Only three patients were nonsmokers. Their ages ranged from 16 to 73 (mean, 57). All patients showed normal or higher levels of serum immunoglobulin before surgery, and all presented positive delayed skin reactions to phytohemagglutinin or tuberculin.

Bacteria. The following bacterial strains were used in this study. *Streptococcus pneumoniae* strains were, for type I, strain A6; for type II, strain 53145; for type III, strain 53146; for type V, strain 5941; for type VIII, strain 594; and for type XII, strain 5943. These *Streptococcus* strains and *Neisseria catarrhalis* A151, *Neisseria flava* 52182, *Neisseria perflava* 52180, *Klebsiella pneumoniae* 52145, and *Haemophilus influenzae* 5152 were obtained from the Pasteur Institute in Paris, France. *Streptococcus* sp. strains A130, C951, D19, 5433, D105, 9, and G4671; *Micrococcus pyogenes* 1060; "*Gaffkya tetragen*" 99; and *Moraxella* sp. strain 1367 were obtained from the Pasteur Institut in Lyon, France.

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After being identified each antigen was lysed separately with Gram stain and complement activation. The resulting suspensions were centrifuged at $20,000 \times g$ for 20 min. The supernatants were discarded, and the pellets were suspended in 10% glycine to a concentration of 15×10 bacteria per ml.

Vaccine. To obtain the vaccine used in this study, the bacteria prepared as previously described were mixed in the following proportions: *S. pneumoniae* types I, II, III, V, VII, and XII, 3.3 ml; *N. catarrhalis*, *N. flava*, and *N. perflava*, 6.6 ml; *Moraxella* sp., 3.3 ml; *K. pneumoniae*, 6.6 ml; *M. pyogenes*, 6.6 ml; and *H. influenzae*, 3.3 ml. After dilution in 100 ml of distilled water, this mixture was filtered twice through a membrane (0.20- μ m pore size). This treatment yielded a clear solution (IRS. 19, Sarback Co., Suresnes 92151, France).

Immunization. Twice a day for 10 days immediately preceding their surgery, 11 patients received 5-ml aerosol doses of the bacterial solution. The last dose was administered 24 h before surgery. One patient suffered acute rhinitis and was consequently excluded from study. The nebulizer used to administer the vaccine was a Monogham ultrasonic nebulizer (Monogham Co., Littleton, Colo.). Investigations show that this device creates a mist with a mean particle size of 5.2 μ m and that it is capable of delivering 35% of a saline solution beyond the carina (6). The other 11 patients were used as controls and were not immunized.

Sample preparation. Samples (pieces of the main stem bronchi, 10 by 40 by 2 mm) were fixed in picric acid-Formalin for 5 h, washed for 12 h in phosphate-buffered saline (PBS; pH 7.2) at 4°C, dehydrated in acetone, and embedded in epoxy plastic (Araldite; CIBA-GEIGY Corp., Summit, N.J.). Sections 1 μ m thick were cut on a hard-tissue microtome (Reichert Autocut) with Ralph-type glass knives (38 mm). Staining was achieved with colloidal iron-periodic acid-Schiff stain after removal of the plastic with sodium methylate.

For immunofluorescence study, better results were obtained with semithin sections after appropriate proteolytic digestion. After being carefully stripped from underlying tissue, pieces (10 by 10 by 2 mm) of mucosa were positioned and snap frozen in tissue tek II (OCT compound, Lab-Tek Products, Div. Miles Laboratories Inc., Westmont, Ill.). Frozen sections, stored at -70°C, were cut to a thickness of 2 μ m in a cryostat (-30°C), mounted on glass slides, and air dried for 5 min before being placed in a moist chamber at a temperature between 18 and 20°C.

Light microscopy. Cell type and mean number of cells were recorded at a magnification of $\times 250$ in 30 adjacent fields containing populations of between 20 and 100 cells. Readings were made first in the lamina propria and then in the submucosa. The Student *t* test was used to determine if there was any relationship between immunization and the total number of cells in the mucosa.

Immunofluorescence microscopy. To detect deposits of immunoglobulin G (IgG), IgA, IgM, IgD, IgE, complement C3, and fibrinogen, direct immunofluorescence with rabbit antihuman sera labeled with fluorescein isothiocyanate (FITC; Boehringer Laboratory, 92500 Rueil Malmaison, France) was carried out as follows. Slides were washed twice in 0.01 M PBS (pH 7.2) for 5 min each time. FITC-labeled antiserum to human serum proteins diluted 1:10 in PBS was applied for 30 min at room temperature. Slides were washed three times in PBS for 5 min each time. To check its validity on tissue known not to contain immunoglobulin-bearing cells, the direct immunofluorescence technique

was applied to bronchial specimens from two healthy victims of accidental death. Light microscopy revealed no plasma cells, and subsequent immunofluorescence staining revealed no immunoglobulin cells.

Indirect immunofluorescence was performed as follows to detect in the mucosa specific antibodies against the following 5 of the 19 bacterial strains: *S. pneumoniae* types II and III, *H. influenzae*, *Streptococcus* sp. strain D19, and *K. pneumoniae*. Slides were submitted to three 5-min washes in PBS. Unlabeled microbial antigen diluted in saline (1:1,000) was applied for 30 min, and then, three more 5-min washes were performed. Monospecific FITC-labeled rabbit microbial serum against *S. pneumoniae* types II and III, *Streptococcus* sp. strain D19, *H. influenzae*, and *K. pneumoniae* were applied for 20 min. Dilution was 1:5 for *S. pneumoniae* types II and III, 1:10 for *Streptococcus* sp. strain D19, and 1:20 for *H. influenzae* and *K. pneumoniae*. Samples were mounted in 90% glycerol in PBS and stored at 4°C pending examination under UV light.

The dilutions of both antisera and antigens were determined by titration with control-positive results. The specificity of each antiserum was checked by immunodiffusion. As a control of the immunofluorescence technique itself, a preliminary study was carried out in both groups on slides pretreated with nonconjugated specific antisera. The absence of staining ascertained the validity of the technique. Bronchial sections from vaccinated and control patients were incubated in a solution containing one of the antigens. The fixed microbial antigen was identified by a second layer of monospecific FITC-labeled serum against the respective bacteria. The mean number of cells bearing each class of immunoglobulin was calculated from readings taken at a magnification of $\times 400$ in the lamina propria and submucosa gland of the three most-populated sections as determined by systematic scanning of whole biopsy specimens. Given the difficulty in identifying ABC in the epithelium, a semiquantitative method of fluorescence analysis had to be used. The technician did not know if the samples came from a vaccinated or immunized subject. To determine if there was any relationship between immunization and the number of ABC, the Wolf g-square test was performed.

RESULTS

Effect of immunization on cell population. Table 1 presents data comparing the number of cells in the immunized and control groups. Plasma cells and lymphocytes were the largely predominating cell types, with polymorphonuclear leukocytes and macrophages accounting for less than 5% of the cell population. The numbers of plasma cells and lymphocytes as well as their ratio to each other varied widely from one location to another. No difference could be found in the overall cell populations in the laminae propriae of immunized and control patients. Conversely, in the submucosa glands, immunized patients exhibited a significantly higher cell population than controls: 62 ± 8 versus 37 ± 5 , respectively ($P < 0.02$). Immunization did not alter the ratio of plasma cells to other cell types in the lamina propria or the submucous glands (Table 1 and Fig. 1).

Immunoglobulin-bearing cells. In both the immunized and control groups, IgA-bearing cells were by far the most frequent at all locations (Fig. 2 and 3). Occasionally, dense IgA staining on the epithelium could not be readily associated with any recognizable immunoglobulin-bearing cells; these areas were excluded from study. Conversely, in the lamina propria and submucous glands, the IgA-bearing cells

TABLE 1. Comparison of the number and types of cells in bronchial mucosa from control and immunized patients^a

Source of cells	No. of fluorescent cells in:		Plasma cell/lymphocyte ratio (%) in:	
	Lamina propria ^b	Submucous glands ^c	Lamina propria ^b	Submucous glands ^b
Control group ^d	50 ± 9	37 ± 5	53 ± 8	62 ± 10
Immunized patients ^d	36 ± 5	62 ± 8	45 ± 8	70 ± 9

^a Means ± standard deviations from the means are presented for 30 adjacent sections at a magnification of ×250.

^b *P*, Not significant.

^c *P* = 0.02.

^d *n* = 11.

were identified as plasma cells. Of the 11 patients in the control group, 7 had no fluorescent cells and 4 displayed at least one cell. In the immunized group, only 3 of 11 patients showed no IgA-bearing cells, and 6 of the 7 patients who displayed fluorescent cells had more than six cells. Only one patient in the control group had six fluorescent cells. This difference in the number of fluorescent cells between controls and immunized patients was statistically significant ($P < 0.05$). The mean counts of cells bearing IgG, IgM, IgD, and IgE were much lower than the count of IgA-bearing cells. Two patients in the control group and three in the immunized group had IgM-bearing cells; in one case there was an equal number of IgA- and IgM-bearing cells. In the control group, one patient had IgG-bearing cells and one had IgD-bearing cells. IgE-bearing cells were not observed.

Specific ABC in the epithelium. Positive responses of the epithelium to the five bacterial strains selected for study were graded on the basis of the intensity of fluorescence. The response was considered weak if fluorescence involved only the surface of the epithelium, mild if the epithelium showed clusters of deep fluorescence, and strong if there were extensive areas of deep fluorescence. In some cases, fluorescence could not be readily correlated to a recognizable ABC (Fig. 4).

For *S. pneumoniae* type II, six patients in the control

group showed no response, and five had weak responses, while in the immunized group, all patients responded positively, with five weak, four mild, and two strong responses. For *S. pneumoniae* type III, seven control patients showed no response, and four had weak responses, while in the immunized group, four patients responded weakly and four responded mildly. For *Streptococcus* sp. strain D19, only one positive reaction was seen in the control group, while six immunized patients responded weakly or mildly. These findings are statistically significant ($P < 0.05$). No patient in either group reacted positively to *H. influenzae* or *K. pneumoniae* (Table 2).

Specific ABC in lamina propria and submucous glands. Immunized patients had significantly more fluorescent *S. pneumoniae* type II ABC than did controls, i.e., 21.6 ± 10 versus 1.2 ± 2 ($P < 0.05$). Similarly, when *S. pneumoniae* type III was studied, immunized patients had 17.9 ± 7 ABC compared with 1.7 ± 1 ABC in controls ($P < 0.05$). As for *Streptococcus* sp. strain D19, the figures were 8 ± 3 in immunized patients and 0.9 ± 12 in controls ($P < 0.05$). Conversely, cells bearing antibodies against *H. influenzae* were rare in both groups, and no *K. pneumoniae* ABC were found. Finally, it should be stated that clusters of fluorescent cells were equally distributed in the epithelium, lamina propria, and submucous glands (Table 3).

FIG. 1. Infiltration by plasma cells (arrows) into the submucous glands of an immunized patient (iron colloidal-periodic acid Schiff stain). Plasma cells were identified by off-centered, heterogenous nuclei.

FIG. 2. Comparison of the number of immunoglobulin-bearing cells per 30 sections (1:400) in lamina propria and submucous glands in control (○) and immunized (●) subjects ($n = 11$). Significant difference between both groups for IgA only, $P < 0.05$.

DISCUSSION

This study was designed to discover whether immunization leads to the appearance of ABC in the bronchial mucosa. Similar studies have been carried out with bronchial washings, but to our knowledge, this is the first time resected bronchi have been examined. The use of bronchial washings has two great shortcomings: the exact origin of the material is ill defined, and serum from inflamed mucosa may cause contamination. In addition to avoiding these two problems, the use of biopsy specimens allowed assessment of ABC even in the submucous glands. In his study of specimens biopsied by means of rigid bronchoscopy, Nijhuis-Heddes obtained submucous glands in only 31 of 42 patients (28). Werner, who also used bronchoscopic biopsies, limited his study to fluorescence in the epithelium and lamina propria (44). Soutar performed his experiments on respiratory tissue obtained during biopsy (37, 38), and thus autolytic changes may have occurred.

All experiments in this study were performed on semithin sections ($1 \mu\text{m}$). At this section thickness, it can be assumed that the cells displaying IgA are the same as those displaying the antigen.

This is thus the first time that observations have been made in the whole bronchus after local immunization either in humans or animals. The submucous glands of immunized patients exhibit more lymphocytes and plasma cells than those of controls, i.e., 62 ± 8 and 37 ± 5 , respectively. It seems safe to assume that this increase is the result of immunostimulation as has been previously reported. La Force, using bronchoalveolar lavage in mice after aerosol immunization with acid-hydrolyzed Re 595 *Salmonella minnesota*, reported an enhancement of cells and, particularly, of polymorphonuclear cells (20, 22). Though it was not seen in our study, an increase in neutrophils has been observed after inhalation of lipopolysaccharides of gram-negative bacteria (12, 36).

FIG. 3. IgA-bearing cells (arrows) in clusters around the submucous glands of an immunized patient (rabbit anti-IgA serum; 1:400).

FIG. 4. Extensive fluorescence of the epithelium (e). Fluorescent plasma cells (arrow) under the epithelium in an immunized patient (rabbit anti-*S. pneumoniae* type II serum; 1:400).

Two factors which may influence immune response and thus must be taken into account are the presence of inflammatory or neoplastic disease and the use of general anesthesia. In an attempt to minimize the importance of these factors, only histologically "normal" tissue was used, and a control group was studied. There is no evidence that lung carcinoma inhibits a patient's immune response (43), although it cannot be ruled out that the response recorded herein would have been still higher in healthy subjects. The only way to resolve this doubt would be to quantify plasma cells throughout the lamina propria and submucous glands.

With regard to the type of immunoglobulin present, a predominance of IgA-bearing cells was recorded in both groups and especially in the immunized group. This finding is in agreement with previous studies using tracheobronchial secretions (30, 39). Kaltreider, who studied the IgA/IgG ratio from the alveoli to the upper airways, reported that the

proportion of IgA increases progressively and is predominant at the carina (18), i.e., the site investigated in this study. Werner (44) carried out a study involving 48 patients biopsied by rigid bronchoscopy before and after a 2-week aerosol vaccination with the same vaccine as ours, and he reported an increase in IgA in the epithelium and in IgA-bearing cells in the lamina propria.

Another question which must be asked concerns the origin of ABC. Are they produced locally by differentiation of a resident precursor, or do they come from extrapulmonary lymphoid tissue? There is no consensus in this area. Several authors have claimed that sensitized lymphocytes can remain in the lung for several weeks, producing an immune response that is entirely independent of systemic immunity (41, 42). Other evidence points to submucosal lymphocytes as a main site of IgA production (23, 40). Finally, recent findings suggest that IgG detected in alveolar lining fluid may be generated at two separate locations (40), but this fact has not been demonstrated for IgA. Our observations of IgA-bearing cells in the lamina propria and submucous glands confirm an endogenous production of IgA but do not rule out an exogenous production. Though it was not done, it would have been interesting to measure antibody levels in serum to know whether or not the local response induces a systemic response; no correlation between aerosol immunization and systemic immune status or bactericidal activity has ever been observed in any of the animal models studied until now (14, 15, 20, 22).

Also relevant to our findings are certain animal studies carried out on nasal or bronchial lavage specimens. In mouse bronchial secretions, Reynolds demonstrated antibody activity in both IgA and IgG secretory fractions (32). In an earlier study, the same author showed that an IgA response occurred in respiratory secretions after inhalation but not after systemic inoculation (31). Rylander, who studied bronchial fluid after a 10-day aerosol immunization with endotoxin of *Escherichia coli*, found that 23% of test animals

TABLE 2. Intensity of fluorescence for specific antibodies in the epithelium

Antimicrobial antibodies tested	No. of patients showing fluorescence ^a							
	Controls				Immunized			
	0	+	++	+++	0	+	++	+++
<i>S. pneumoniae</i> type II ^b	6	5			0	5	4	2
<i>S. pneumoniae</i> type III ^b	7	4			3	4	4	
<i>Streptococcus</i> sp. strain D19 ^b	10	1			5	4	2	
<i>H. influenzae</i>	11				11			
<i>K. pneumoniae</i>	11				11			

^a 0, Negative; +, surface; ++, clusters of deep fluorescence; +++, extensive areas of deep fluorescence. *n* = 11 for control and for immunized groups.

^b *P* < 0.05.

TABLE 3. Mean number of ABC in lamina propria and submucous glands^a

Antimicrobial antibodies tested	Mean no. $\pm$ standard deviation of ABC in:	
	Controls ^b	Immunized patients ^b
<i>S. pneumoniae</i> type II ^c	1.2 $\pm$ 1	21.6 $\pm$ 10
<i>S. pneumoniae</i> type III ^c	1.7 $\pm$ 1	17.9 $\pm$ 7
<i>Streptococcus</i> D19 ^c	0.9 $\pm$ 1	8 $\pm$ 3
<i>H. influenzae</i>	0.7	1.1
<i>K. pneumoniae</i>	0	0

^a Three sections, $\times 400$.^b $n = 11$.^c $P < 0.05$.

exhibited IgG antibodies and 88% exhibited IgA antibodies (34).

In the epithelium of immunized patients, specific fluorescence was observed for three of the five antigens studied, i.e., for *S. pneumoniae* types II and III and for *Streptococcus* sp. strain D19. Corresponding increases in the number of cells bearing antibodies for these antigens were also recorded in the lamina propria and submucous glands.

As previous authors have stated (28, 38, 39), counting of fluorescent cells is difficult because of clustering. Nevertheless, ABC for *S. pneumoniae* types II and III in immunized patients so greatly outnumber those in controls, 20 to 1 and 17.9 to 1.7, respectively, that there can be no doubt that the difference is significant, regardless of the counting or staining method. Furthermore, so large a gap suggests a booster effect rather than a genuine immunization. This assumption is justifiable for two reasons. First, this study dealt with older patients with chronic colonization of their airways due to chronic inflammatory bronchial disease. Second, the bacteria studied included some of the strains most commonly observed in the adult European population, since *S. pneumoniae* types II and III are ranked 14th and 4th, respectively, in French pneumococcal serotyping ratings (7).

Given its ranking, *S. pneumoniae* type III should logically have induced a higher immune response than it did in the control group. This inconsistency may stem from alterations in the normal flora caused by antibacterial therapy prior to vaccination or to colonization of the respiratory tract by nosocomial gram-negative rods (21). Another surprising observation was that cells bearing antibodies against *K. pneumoniae* were not found after immunization. *K. pneumoniae* is highly immunogenic, and its presence along with *S. pneumoniae* types II and III has been previously reported (44). A possible explanation for this inconsistency is that *K. pneumoniae* polysaccharides act as polyclonal B-cell activators rather than specific antibody stimulators; this activity has been shown in mice (27) and in humans (9).

Using a bacterial vaccine in the respiratory tract can be dangerous because of the presence of gram-negative organisms which produce endotoxins leading to pulmonary toxicity, pyrogenicity, and granulocyte recruitment (10, 12, 36). In this study, one adverse reaction (acute rhinitis without fever) was recorded. The lipid A portion of lipopolysaccharide is generally thought to mediate this endotoxic activity (3) probably by both direct (24, 45) and indirect (26, 45) complement activation. A typical response after exposure to gram-negative bacteria or endotoxin (3, 10) is the migration of neutrophils into the airways. In our model, histological sections showed no enhancement of neutrophils (data not

shown); this result suggests that the vaccine either is devoid of lipopolysaccharide or, at least, contains no biologically active endotoxin (36).

This study is interesting for several reasons. The first is that it describes a new model for investigating human immune response to a local challenge in the respiratory tract, i.e., in vivo immunization of patients scheduled for pneumectomy with an antigen extract, followed by histological examination and fluorescence assay of the surgical pieces. It is also interesting because of its results, which not only confirmed earlier reports, but also supplied greater insight. As previously reported, immunization was seen to bring on an enhancement of plasma cells. In immunized patients, substantially more IgA and IgA-bearing cells were found than in controls. By contrast, few IgM- or IgG-bearing cells were detected in either group. The new finding of this report is that aerosol immunization can lead to specific antibody production, in this case against *S. pneumoniae* types II and III and *Streptococcus* sp. strain D19 by IgA-class plasma cells.

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