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Antibody Response to *Prevotella* spp. in Patients with Ventilator-Associated Pneumonia

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Although anaerobic bacteria are frequently isolated from the oropharyngeal flora, their potential pathogenic role in ventilator-associated pneumonia (VAP) has been poorly investigated. In order to evaluate the pathogenic role of *Prevotella* spp. isolated from protected specimen brushes, we investigated the systemic humoral response with the enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot) in 13 patients who developed a VAP associated with *Prevotella* species (group I). The antigen used was a mixture of whole-cell proteins taken from four reference *Prevotella* strains. We compared the antibody levels observed in these patients with those measured in 30 patients who developed a VAP unrelated to anaerobic bacteria (group II), in 27 patients with dental stumps (group III), and in 30 healthy patients (group IV) who had *Prevotella* species on dental plaque. The ELISA levels obtained in the four groups showed significant differences between group I and each of the three control groups ($P < 0.05$). The antibody profiles obtained by Western blot showed an intensity of response roughly superposable over levels obtained by ELISA and a species specificity. These findings suggested that colonization of these patients with *Prevotella* species may have been associated with an infectious process leading to a systemic humoral response and that these bacteria could play a role in VAP.

Most species of the genus *Prevotella* are important members of the normal mouth flora. These bacteria have been isolated from periodontal lesions and have also been associated with lung infections, especially secondary to aspiration (2, 3, 15). Nosocomial pneumonia, occurring in mechanically ventilated patients, is usually associated with a high mortality rate despite the institution of broad-spectrum antibiotic therapies (5, 12, 13). The high prevalence of nosocomial pneumonia may be related to modifications of the oropharyngeal flora of the critically ill patients and particularly to colonization by gram-negative bacilli (17). Numerous studies have reported that nosocomial pneumonias are mainly due to gram-negative bacteria and to *Staphylococcus aureus* (6, 12, 22). Although anaerobic bacteria have been isolated from the oropharyngeal flora (15), their pathogenic role in ventilator-associated pneumonia (VAP) has been poorly investigated. Isolation of anaerobic bacteria requires adequate transport conditions and specific growth media. This may not be systematically achieved during bacteriological investigation in VAP. In the last few years, we studied 133 patients suffering from a first episode of VAP who were sampled by using protected specimen brushes (PSB). We showed that 76% of these specimens yielded aerobic bacteria and 24% yielded at least one anaerobic bacterial strain (9). Fifty-five percent of the isolated anaerobic strains belonged to the genus *Prevotella*. Nevertheless, because of the risk of contamination of the samples, the role of these bacterial strains remains questionable and additional evidence is required to determine their responsibility in the current infection. It may reasonably be hypothesized that bacteria causing VAP originate from the oropharyngeal flora. Because anaerobic bacteria are well known to be predominant in this flora, they are most likely to be responsible for the infection and may be considered first. It is commonly acknowledged that bacteria involved in an infectious process elicit a strong immune response. Thus, in order to evaluate the pathogenic role of the *Prevotella* spp. isolated from PSB, we investigated the systemic humoral response in patients who developed VAP associated with *Prevotella* species. We compared the serum antibody levels observed in these patients with those measured in patients who developed a VAP unrelated to anaerobic bacteria, in patients with dental stumps, and in healthy patients.

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MATERIALS AND METHODS

Patients. We conducted a prospective study from December 1992 to February 1995. Four groups of individuals were investigated in this study. Group I comprised a group of 13 patients (mean age ± standard deviation: 58.8 ± 16.3 years) with VAP, hospitalized in the medical intensive care unit, in whom *Prevotella* species, associated with other bacteria or alone, were isolated from PSB. Group II was a control group of 30 patients (mean age, 61.9 ± 17.8 years) with VAP hospitalized in the same intensive care unit but in whom no anaerobic bacteria were isolated from PSB. Group III was another control group of 27 patients (mean age, 78.7 ± 9.3 years) with dental stumps but without pulmonary infection. Group IV was another control group of 30 healthy volunteers (mean age, 35.6 ± 7.3 years) in whom *Prevotella* species were isolated from dental plaque. These subjects had not had any dental care within the previous 15 days.

Clinical investigations. The diagnosis of pneumonia was based on recent and persistent (>24 h) infiltration on chest radiograph and at least two of the following clinical criteria: fever (>38.3°C) or hypothermia (<36.5°C), macroscopically purulent tracheal aspirates, and leukocytosis (leukocytes, $>10 \times 10^9$/liter) or neutropenia (leukocytes, $<4 \times 10^9$/liter). Pneumonia was considered a VAP when it occurred after 48 h of mechanical ventilation and was judged not to have had an incubation period before the outset of mechanical ventilation.

Fiberoptic bronchoscopic examinations were performed in accordance with the recommendations for the standardization of bronchoscopic techniques (20). Before the procedure, the bronchoscope (Olympus P20 D) was cleaned by complete immersion for 15 min in a basic glutaraldehyde solution (Cidex; Johnson Johnson, Viroflay, France), rinsed with sterile water, and then stored in a sterile package. The bronchoscope was introduced through an adapter to minimize air leaks and was led to the bronchial orifice of a lung segment according to the location of pulmonary infiltrates on the chest radiograph. No topical anesthetics were used. A telescopic catheter-protected brush with a distal polyethylene glycol occlusion (Microvasive; Boston, Mass.) was inserted through the inner suction channel. The PSB was advanced in the fiberoptic bronchoscope to a wedged peripheral position. Then the inner cannula was protruded to eject the distal carbon wax plug, and the catheter was advanced to collect secretions of the

lower airway tract for microbial cultures. After the sample was obtained, the brush was retracted into the inner cannula, and then the catheter was removed from the bronchoscope. The brush was advanced and aseptically cut into a sterile tube containing 1 ml of Schaedler broth (BioMérieux, Lyon, France); the presence of reduced cysteine in this transport medium provides an oxidoreductant system and decreases the exposure of bacteria to oxygen. Specimens were transported to the laboratory within 15 min.

Bacteriologic examination. The Schaedler broth containing the brush was gently vortexed. Aliquots of 10 μl were spread on two agar medium plates prepared within the previous 72 h: meat-yeast agar (VL Sanofi Pasteur, Marnes la Coquette, France) enriched with sheep blood (5%) and another plate of the same medium containing gentamicin (40 μg/ml). Liquid Rosenow's medium (Sanofi Pasteur) containing cysteine hydrochloride was also used to isolate certain anaerobes suspected on direct examination and not obtained on solid media. These plates were immediately incubated under anaerobic conditions (Anaerocult; Merck, Darmstadt, Germany) at 37°C. Then the Schaedler broth was thoroughly vortexed to suspend all material from the brush. Aliquots of 10 μl were inoculated onto standard media for isolation of aerobic bacteria. After 72 h of incubation, bacterial growth $>10^2$ CFU/ml was detectable.

Plaque swabbings of the group IV healthy controls were inoculated onto selective néomycine-vancomycin-Schaedler agar (BioMérieux) in order to isolate *Prevotella* species.

Anaerobic bacteria were identified by using the API 20A rapid ID32A system (BioMérieux) and by identifying the fermentation products by gas-liquid chromatography (Perkin-Elmer, Saint-Quentin, France) (16, 25). The production of β-lactamase by gram-negative rods was determined by the Cefinase nitrocefin method (BBL Becton Dickinson, Cockeysville, Md.).

Blood samples. For groups I and II, blood samples were collected within 15 days following the PSB procedure. For group III, serum samples were collected during medical consultation. For group IV, serum samples were collected at the time of plaque swabbing. After blood centrifugation, sera were stored at −20°C before use.

ELISA. For enzyme-linked immunosorbent assay (ELISA), an antigenic preparation was obtained from four reference strains: *Prevotella intermedia* ATCC 25611, *Prevotella melaninogenica* ATCC 25845, *Prevotella oralis* ATCC 33269, and *Prevotella buccae* ATCC 33574. The four reference *Prevotella* strains were cultured under anaerobic conditions at 37°C on VL agar (Sanofi Pasteur) supplemented with 5% sheep blood. The organisms were harvested by swabbing and washed twice in sterile 0.15 M NaCl. The bacteria were then suspended in 3 ml of sterile distilled water and sonicated for 5 min at 50% duty cycle and with the output control on 5 (Branson Sonifier 450; OSI, Marepas, France). The suspensions were then centrifugated (14,000 × *g*; 10 min). Protein concentrations of the supernatants were determined by the bicinchoninic acid method (Pierce Chemicals, Rockford, Ill.). These antigenic solutions were kept frozen at −80°C.

Microtitration plates (Maxisorp Nunc; Polylabo, Strasbourg, France) were coated with the antigenic preparation, 100 μl per well, diluted in 0.05 M carbonate buffer (pH 9.6) at a concentration of 10 μg/ml (0.25 μg of protein per well for each reference strain). The plates were stored at 4°C for 24 h. Unsaturated binding sites were then blocked for 24 h at 4°C with 200 μl of phosphate-buffered saline (PBS; pH 7.2) per well containing 10 mg of bovine serum albumin (Sigma) per ml and 0.05% Tween 20 (Sigma). Before use, the plates were washed three times in PBS containing 0.05% Tween 20, using a plate washer (LP 35; Sanofi Pasteur). All sera were tested in triplicate (100 μl per well) at a 1/500 dilution in PBS-Tween buffer containing 0.1% gelatin (Sigma) and 0.5% bovine gamma globulin (Sigma). To determine the specificity of the antibody responses to the antigenic preparation, adsorption of every serum sample with this antigenic preparation was performed as follows: 200 μl of each serum sample diluted 1/250 was added to 200 μl of antigenic preparation at a concentration of 100 μg (25 μg of each bacterium) per ml of carbonate buffer (pH 9.6) for 1 h at 37°C. After 10 min of centrifugation (10,000 × *g*), the resulting 1/500 supernatant dilutions were used to test for residual activity (tested in triplicate). A reference serum used to standardize the ELISA was selected on the basis of dilution curves. After the sera were added, plates were incubated for 1 h at 37°C and washed three times in PBS-Tween. Peroxidase-labelled rabbit anti-human immunoglobulin A (IgA), IgM, and IgG (Dakopatts, Copenhagen, Denmark) were each diluted 1/1,000 in PBS-bovine serum albumin-Tween-bovine gamma globulin and added to each well (100 μl). The plates were then incubated again for 1 h at 37°C and washed again as described above. Freshly prepared substrate solution (0.05% hydrogen peroxide; Sigma) with 1 mg of ABTS [2.2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma] per ml in McIlvain's buffer (pH 4.6) was added to each well (100 μl) (14). After 30 min at room temperature, the color reactions were read in the plate reader (Anthos Labtec Instruments, Salzburg, Austria) at 414 nm.

We expressed the results as arbitrary ELISA units calculated by comparison with a reference serum according to the following formula: $U = [(OD_x - OD_{xa})/(OD_{ref} - OD_{refa})] \times 100$, where OD_x and OD_{ref} were the optical densities (OD) observed (mean of three wells) for the tested and reference serum samples, respectively; and OD_{xa} and OD_{refa} were the OD recorded after adsorption on the antigenic preparation (1). Specific IgM antibody levels were assessed for patients belonging to group I (VAP patients "infected" with *Prevotella* sp.) and for patients belonging to group IV (healthy *Prevotella* carriers). Sera were tested in triplicate at a 1/100 dilution. Peroxidase-labelled rabbit anti-human IgM (Sanofi Pasteur) was diluted 1/1,000. Results were expressed as arbitrary ELISA units calculated by comparison with a reference serum used previously.

Western blot. To further define the antibody patterns of the antibody-positive subjects, we performed a Western blot (immunoblot). We used the antigenic preparation and the same serum dilution (1/500) as we used for ELISA. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the antigenic preparation was performed according to the method of Laemmli (18) with a 4% stacking gel and a 10% separating gel. We used a mini-gel apparatus (Mini-Protean II dual slab gel; Bio-Rad Laboratories). Prior to electrophoresis, the antigen was heated at 100°C for 5 min in Tris-HCl buffer (pH 6.8) containing 1% SDS–10% 2-mercaptoethanol. Electrophoresis was conducted under a constant voltage of 25 V/cm (Pharmacia LKB Multidrive XL power supply) for 1 h. Proteins were blotted for 1 h onto prewetted nitrocellulose membranes (Bio-Rad) by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) under a constant current of 200 mA. The blots were incubated for 1 h with human sera diluted to 1/500. They were rinsed three times in Tris-saline blotting buffer (pH 8) and incubated for 1 h in alkaline phosphatase-conjugated anti-human IgA, IgG, and IgM (Dakopatts). After the nitrocellulose sheets were washed, they were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP) as the substrate and with nitroblue tetrazolium as the chromogenic indicator. The reactions were stopped after 20 min by washing the filters extensively with distilled water.

Statistical analysis. ELISA OD were log transformed to achieve normality and homoscedasticity of the distributions among the four groups. The null hypothesis of no difference of the mean immune status between groups was tested by a one-way analysis of variance, followed, in case of rejection, by Fisher's least-significant-difference *t* tests for localizing what means actually differ. All comparisons were considered significant at the 0.05 level. Computations were performed with SAS/STAT software for PC release 6.04.

RESULTS

Bacteriological data. During the study period, 111 patients were suspected of VAP. Sixty patients (54%) had a positive culture by PSB: 45 (75%) with only aerobic bacteria, 13 (22%) with aerobic and anaerobic bacteria, and 2 (3%) with only anaerobes. Fifteen patients had anaerobic bacteria, representing 13.5% of the whole and 25% of the bacteriologically positive VAP cases. *Prevotella* species were isolated in 13 of these 15 patients (87%). The following 25 anaerobic strains were isolated: *P. melaninogenica* (5 strains), *P. intermedia* (4 strains), *P. buccae* (4 strains), *P. oralis* (2 strains), *P. loescheii* (2 strains), *Fusobacterium nucleatum* (3 strains), *Eubacterium lentum* (1 strain), *Veillonella* sp. (1 strain), *Peptostreptococcus anaerobius* (1 strain), *Peptostreptococcus micros* (1 strain), and *Peptostreptococcus prevotii* (1 strain). The associated aerobic bacteria were as follows: *Pseudomonas aeruginosa* (3 strains), *Acinetobacter baumanii* (3 strains), enterobacteria (3 strains), *Streptococcus "milleri"* group (3 strains), *Staphylococcus aureus* (2 strains), *Streptococcus* sp. F group (2 strains), *Streptococcus pneumoniae* (1 strain), *Haemophilus influenzae* (1 strain), and *Legionella pneumophila* (1 strain).

The culture of dental plaque (group IV) demonstrated that all persons sampled yielded *Prevotella* species (Table 1). Sixty strains were isolated: 22 *P. buccae*, 10 *P. melaninogenica*, 9 *P. intermedia*, 10 *P. oralis*, 5 *P. denticola*, 3 *P. loescheii*, and 1 *P. buccalis* strain.

Serum antibody response to *Prevotella* species. To determine whether isolation of *Prevotella* species on PSB was associated with a humoral antibody response, patient sera were tested by ELISA with an antigenic preparation that included all antigens of the more frequently isolated *Prevotella* species. The antibody levels obtained in the four groups are shown in Table 1. The levels observed in the sick patients of group I were compared with those seen in the three control groups and are shown in Fig. 1. Analysis of variance, after log transformation, found a significant difference between the four groups ($P = 0.031$). Fisher's least-significant-difference *t* tests showed significant differences between group I and each of the three control groups ($P < 0.05$), whereas no significant differences were found between groups II, III, and IV. The latter result was confirmed by an analysis of variance between the three

TABLE 1. Antibody levels against *Prevotella* spp. exhibited by individual patients and bacteriological data from study groups I and IV[a]

Patient	Group I			Group II total antibody level	Group III total antibody level	Group IV		
	Antibody level		*Prevotella* sp. on distal brush			Antibody level		*Prevotella* sp. on dental plaque
	Total	IgM				Total	IgM	
1	145.9	1.4	*P. melaninogenica*	50.0	5.0	18.9	1.3	*P. intermedia*, *P. melaninogenica*
2	33.5	0.1	*P. melaninogenica*	9.8	11.0	23.7	2.8	*P. buccae*, *P. melaninogenica*
3	5.5	0.8	*P. melaninogenica*	5.9	24.9	24.8	2.0	*P. buccae*
4	30.5	0.5	*P. oralis*	33.5	22.3	8.8	4.0	*P. loescheii*, *P. oralis*, *P. buccae*
5	17.1	6.1	*P. buccae*	17.0	94.0	38.3	3.0	*P. oralis*
6	143.1	4.5	*P. intermedia*, *P. buccae*, *P. melaninogenica*	2.1	13.5	12.8	4.8	*P. denticola*
7	93.2	6.1	*P. intermedia*, *P. melaninogenica*	28.0	43.9	23.2	2.9	*P. buccae*, *P. intermedia*, *P. oralis*
8	37.7	3.6	*P. buccae*, *P. intermedia*	8.8	39.1	16.3	5.5	*P. buccae*, *P. loescheii*
9	52.2	4.9	*P. buccae*	102.5	84.8	31.5	3.7	*P. oralis*, *P. buccae*
10	48.1	0.2	*P. loescheii*	83.1	1.6	22.2	1.9	*P. buccae*
11	96.7	1.2	*P. intermedia*	9.7	22.2	48.4	2.7	*P. melaninogenica*, *P. oralis*, *P. denticola*
12	57.7	0.5	*P. oralis*	4.7	26.7	14.8	2.2	*P. loescheii*, *P. intermedia*
13	52.2	10.8	*P. loescheii*	17.9	19.7	39.6	5.0	*P. melaninogenica*, *P. denticola*, *P. buccae*
14				20.6	7.2	77.6	7.7	*P. melaninogenica*, *P. oralis*
15				27.1	9.6	21.7	3.9	*P. oralis*, *P. buccae*, *P. intermedia*
16				7.8	36.6	14.8	10.6	*P. oralis*, *P. buccae*, *P. intermedia*
17				16.0	31.0	95.2	5.8	*P. buccae*, *P. intermedia*
18				20.0	19.6	57.8	6.7	*P. melaninogenica*, *P. buccae*
19				29.0	6.1	6.7	2.3	*P. denticola*, *P. buccae*
20				7.7	47.7	22.6	4.5	*P. buccalis*
21				15.3	67.0	24.9	3.2	*P. buccae*, *P. melaninogenica*, *P. intermedia*
22				29.8	55.0	19.5	1.5	*P. oralis*, *P. buccae*
23				15.7	9.9	60.2	3.3	*P. melaninogenica*, *P. denticola*
24				72.6	44.0	42.4	7.4	*P. buccae*, *P. melaninogenica*, *P. intermedia*
25				47.0	31.0	49.3	7.0	*P. oralis*, *P. buccae*, *P. intermedia*
26				62.6	67.8	8.2	3.3	*P. buccae*
27				41.1	16.5	17.4	2.0	*P. buccae*
28				9.2		8.7	0.6	*P. melaninogenica*, *P. denticola*, *P. buccae*
29				52.6		9.5	1.8	*P. buccae*, *P. melaninogenica*
30				13.6		82.2	3.7	*P. buccae*

[a] See Materials and Methods for composition of study groups. Antibody levels are expressed in arbitrary ELISA units (see text).

control groups. There was a highly significant difference between group I and the aggregate of groups II, III, and IV ($P = 0.005$) by analysis of variance.

To determine whether the immunologic exposure to *Prevotella* antigen was recent, we compared levels of specific IgM for patients of groups I and IV. Results are displayed in Table 1. The means ± standard deviations are 3.1 ± 3.2 and 3.9 ± 2.2 arbitrary ELISA units for groups I and IV, respectively. Analysis of variance resulted in a nonsignificant difference between group I (sick patients) and group IV (healthy patients).

Results of Western blot. The antibody profiles observed for the four groups were more complex by Western blot, with sera having higher antibody levels (data not shown). We compared the resulting group I and IV antibody profiles with the bacteriological culture data in order to determine whether they were characteristic of a *Prevotella* species. We observed that the most complex profile was associated with *P. melaninogenica*: group I patients 1, 2, 6, and 7 and group IV patients 14 and 23 (Fig. 2). *P. intermedia* was associated with a smear in the molecular weight zone above 67,000: group I patients 6, 7, and 11 and group IV patient 24. A single band close to 43 kDa was observed with *P. buccae*: group I patients 5 and 9 and group IV patients 10, 26, and 28. We could not see any characteristic profile for *P. oralis.*

DISCUSSION

Although Bartlett found anaerobic bacteria in 35% of hospital-acquired pneumonia cases (3), the role of anaerobes in patients with VAP is not well established (4). In VAP patients, anaerobic bacteria are seldom isolated in PSB, probably because they require adequate transport and culture conditions which are not systematically achieved during bacteriological investigation. The aim of this study was to evaluate the pathogenic role of anaerobes in VAP. As bacterial infections are frequently associated with an immune response specific to the pathogenic microorganisms, we investigated the systemic humoral response in patients who developed a VAP associated with *Prevotella* species in PSB. We studied *Prevotella* species because they were the most frequent of the various anaerobic

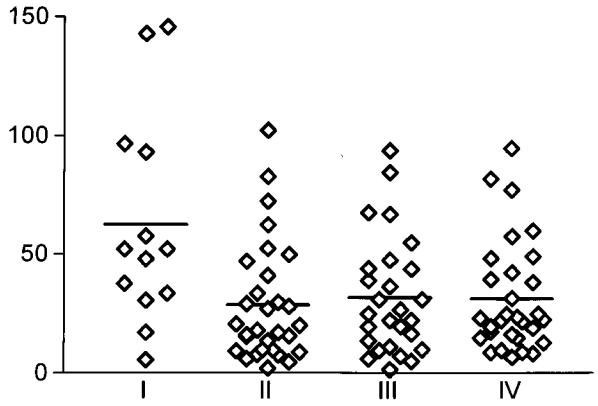

FIG. 1. Distribution of total antibody levels in the four study groups. Horizontal bars represent the means. Means (± standard deviations) were as follows: 63 ± 45, 29 ± 25, 32 ± 25, and 31 ± 23 arbitrary ELISA units for groups I, II, III, and IV, respectively.

bacteria isolated on PSB. Antibody responses to *Bacteroides* species (now named *Prevotella* [24]) have been examined in studies of human periondontal diseases (11), but no publications have reported antibody responses against these species in pulmonary infections.

All persons sampled in the control group of healthy volunteers (group IV) had *Prevotella* species on dental plaque. The species isolated most frequently was *P. buccae*. This group was studied to determine whether colonization of the dental plaque by these anaerobic bacteria leads to a specific immune response. In these patients, we observed that the presence of these bacteria was rarely associated with high antibody levels. Danielsen et al. (7) observed that responses might be due to a gingival lesion through which microorganisms were introduced into the tissue, eliciting an immune response. It is also possible that these patients have an asymptomatic infection due to *Prevotella* spp.

Group III was studied to analyze whether patients with dental stumps could produce antibodies against *Prevotella* spp. Apparently, dental decay did not significantly increase antibody levels against these bacteria. Ebersole et al. showed that *Porphyromonas gingivalis* is the anaerobic bacterium most frequently implicated in periodontal diseases (11). They demonstrated a higher level of antibody to *Porphyromonas gingivalis* in patients suffering from periodontitis or from advanced destructive periodontitis than in all other diseased or healthy individuals studied. Among the *Prevotella* species, only *P. intermedia* was significantly associated with advanced destructive periodontitis patients compared with other diseased or healthy persons. With the other black-pigmented *Prevotella* spp., including *P. melaninogenica* and *P. loescheii*, and the nonpigmented *Prevotella* spp., such as *P. oralis* and *P. buccae*, they observed some extreme responses, but the distribution of responses was independent of disease classification. Persson et al. demonstrated that immunization against *Porphyromonas gingivalis* inhibits progression of experimental periodontitis in nonhuman primates, confirming the important role of this species in dental pathology (21). Our results for group III show no significant difference from those for group IV made up of healthy people. This confirms the minor role of *Prevotella* species in periodontal diseases compared with the role of *Porphyromonas gingivalis*. However, patients of group III are older than those of the other groups, and it is well known that acquired local immune response and systemic immune response decrease with age.

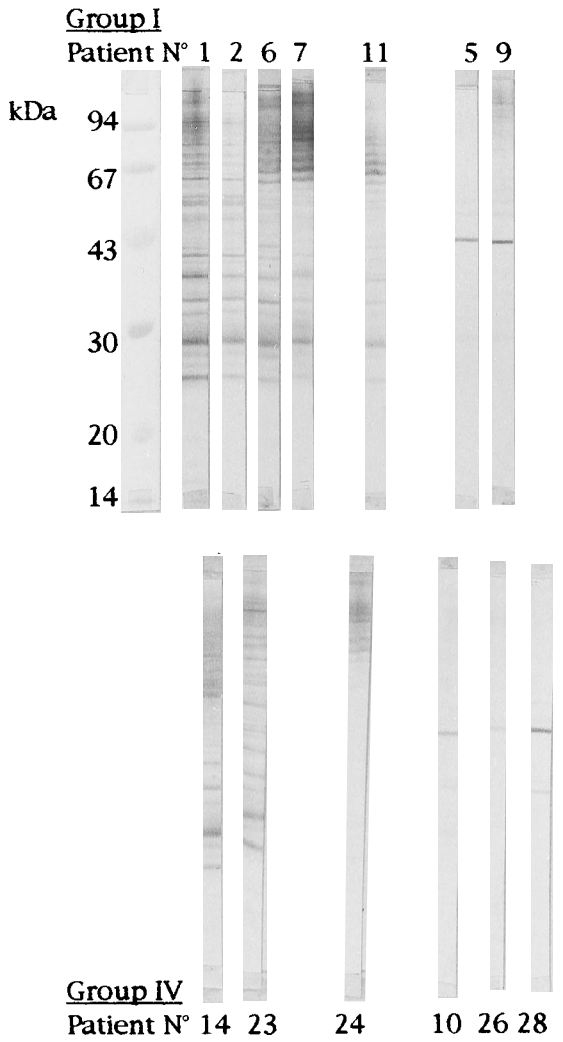

FIG. 2. Immunoblot demonstrating specificity of antibody response against *Prevotella* antigens in patients of groups I and IV. Molecular weight standards were stained with Ponceau S. *P. melaninogenica* was isolated on distal brushings of patients 1, 2, 6, and 7 of group I and on the dental plaque of patients 14 and 23 of group IV. *P. intermedia* was isolated on distal brushing of patients 6, 7, and 11 of group I and on the dental plaque of patient 24 of group IV. *P. buccae* was isolated on distal brushing of patients 5 and 9 of group I and on the dental plaque of patients 10, 26, and 28 of group IV.

Group II included patients clinically similar to those of group I (sick patients). They were hospitalized in the same intensive care unit. They also had VAP but without isolation of anaerobic bacteria from PSB. The mean antibody level to *Prevotella* antigens was not statistically different from those obtained in the control groups III and IV.

All diseased patients of group I had *Prevotella* species in their distal brushing at a concentration of $>10^2$ CFU/ml of broth. At a recent international consensus conference on VAP, a threshold of 10^3 CFU/ml for PSB was accepted as a probable indication of etiologic role (4). However, this threshold was established for aerobes and there was no consensus for anaerobic strains. We chose the threshold of 10^2 CFU/ml because of the fragility of most anaerobic bacteria. Furthermore, Dreyfuss et al. noted that 30% of patients with a PSB culture of $>10^2$ CFU/ml but $<10^3$ CFU/ml ultimately developed colony counts indicative of pneumonia (10). In our study, *P. melaninogenica* and *P. intermedia* were the most frequently isolated species, as

observed by Marina et al. (19) and Bartlett (3). The mean antibody level in this group is statistically different from that in the control groups ($P < 0.05$). Because the mean antibody levels in the three control groups were not statistically different ($P = 0.69$), we could aggregate them into a single control group. Thus, there was a highly significant difference between group I, made up of diseased patients, and the aggregate of the control groups ($P = 0.005$). These findings suggest that colonization of these patients with *Prevotella* species may have been associated with an infectious process leading to a systemic humoral response and that these bacteria could play a role in VAP. The results of IgM titration suggest that in most of the patients of group I the immune response is a secondary response lacking IgM. This suggests that the current *Prevotella* infection is not their first *Prevotella* infection. They may have had previous dental infections with these bacteria.

By the Western blot method, the intensity of the response was at first glance roughly superposable to levels obtained by ELISA. Marked responses were observed for the highest antibody levels; conversely, no band was observed in patients who had a low antibody level (data not shown). However, we found some apparent discrepancies, generally in middle levels around 30 units. In these cases, ELISA levels were always higher than suggested by immunoblot results. Thus, we think that the discrepancies are due to antibodies against antigens which are not proteic in nature and are not visible on immunoblots (8).

P. melaninogenica and *P. intermedia* seem to be the most antigenic of the four species tested. We did not incorporate *P. loescheii* into our antigenic preparation because we thought the antigenic cross-reactions could lead to an antibody response against neighboring species. The immunoblots show a species specificity in the antibody response, and it is possible that the response observed in some patients would have been greater if *P. loescheii* had been incorporated into the antigenic mixture.

In this study, we found that the occurrence of VAP associated with anaerobic bacteria (*Prevotella* species) isolated from PSB is associated with an antibody response which seems specific to the different *Prevotella* species. All data favor the pathogenic role of anaerobes, especially *Prevotella* species, in VAP.

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