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Hemin-Induced Modifications of the Antigenicity and Hemin-Binding Capacity of *Porphyromonas gingivalis* Lipopolysaccharide

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Previous studies have shown that the physical, biochemical, and antigenic properties of the bacterial outer membrane are profoundly influenced by the growth environment. In the present study, the effects of growth in hemin-replete (H+) and hemin-depleted (H−) media on the lipopolysaccharide (LPS) of the oral pathogen *Porphyromonas gingivalis* were investigated. Our studies show that LPS from *P. gingivalis* cultured in H+ media (H+LPS) expressed additional low-molecular-mass antigens, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. Particularly evident was a 26-kDa antigen (26 LPSC) that was lost from the LPS upon transfer of *P. gingivalis* into H− media. The loss of the 26 LPSC was accompanied by a marked reduction in the hemin-binding capacity of the LPS. The 26 LPSC was refractory to Coomassie blue staining and proteinase K digestion. H+LPS from strain W50/BE1, a nonpigmented pleiotropic strain, lacked the 26 LPSC and did not bind hemin. Polyclonal antiserum raised to whole-cell antigens of *P. gingivalis* A7436, W83, and HG405 grown in H+ media, but not in H− media, recognized the 26 LPSC in the purified H+LPS from any of the three strains. The immunoreactivities of sera from humans with ($n = 24$) or without ($n = 25$) periodontitis to the 26 LPSC and other H+LPS determinants were analyzed by Western blot. Overall, 75% of adult periodontitis patient sera reacted with multiple bands in the H+LPS stepladder, particularly in the range of 14 to 27 kDa. In contrast, only 20% of control sera reacted faintly with H+LPS bands in the range 27 to 34 kDa. The 26 LPSC was recognized by over 40% of sera from adult patients with periodontitis and none of the healthy control sera. Taken together, these results suggest that the antigenicity and hemin-binding properties of *P. gingivalis* LPS can be modified by growth in H+ media.

Lipopolysaccharides (LPS) are among the most potent cell agonists known: LPS can act at picomolar concentrations to trigger host inflammatory cells to release toxic oxygen radicals, arachidonic acid derivatives, and cytokines (20). These products can in turn initiate a cascade of pathophysiological events that can culminate in septic shock and tissue destruction (30). The LPS of *Porphyromonas* (formerly *Bacteroides*) *gingivalis*, an opportunistic oral pathogen, has relatively little proinflammatory activity according to some reports (for a review, see reference 16), while other reports suggest that it has potent proinflammatory activity (6). These contradictory reports may relate to different growth conditions and to different methods of LPS purification. *P. gingivalis* is subjected to many environmental changes in the gingival crevice during periodontal disease activity, including a decrease in the redox potential (21), an increase in gingival bleeding on mild stimulation (11), and an increase in crevicular fluid iron (up to 170 μM iron) (22). Much of the crevicular fluid iron is thought to be derived from the hemin molecule (13). While the iron atom in the hemin molecule is the critical constituent for growth, the protoporphyrin IX ring has been suggested to play a role in the binding and transport of iron into the cell (13). Modifying the hemin concentration in the growth medium has been shown to modulate the virulence of *P. gingivalis*, presumably through alterations in outer membrane protein expression (3); however, the effects of hemin on *P. gingivalis* LPS have not been examined. Interestingly, the LPS of several bacterial species (2, 24), including *P. gingivalis* (13), can bind hemin and may play a role in hemin sequestration and storage (26).

In the present study, the effects of growth of *P. gingivalis* in hemin-replete (H+) media and hemin-depleted (H−) media on specific physical, biochemical, and antigenic properties of *P. gingivalis* LPS were examined. Our results indicate that growth of *P. gingivalis* in H+ media results in the expression of an additional 26-kDa moiety (the 26 LPSC). The 26 LPSC is refractory to Coomassie blue staining and proteinase K digestion, is immunogenic, and is recognized by serum antibodies from periodontitis patients but not healthy control subjects. The 26 LPSC was lost upon transfer of *P. gingivalis* into H− media, which was accompanied by a decrease in the hemin-binding capacity of the LPS.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* A7436, a clinical isolate originally characterized by the late V. R. Dowell (Anaerobic Laboratory, Centers for Disease Control and Prevention, Atlanta, Ga.), was the principal strain used in this study. Also employed in the LPS studies were strains W83, ATCC 33277 (American Type Culture Collection, Rockville, Md.), W50, W50/BE1 (kindly provided by A. S. McKee, PALS, Porton Down, United Kingdom), HG 405, HG 864 (kindly provided by A. J. Van Winkelhoff, Academic Center for Dentistry, Amsterdam, The Netherlands), and the transpositional mutant strain MSM-3, which has a defect in the transport and utilization of heme (13). Stock cultures were maintained on anaerobic blood agar plates (Remel, Lenexa, Kans.) in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) at 37°C in an atmosphere of 85% N_2–5% H_2–10% CO_2. The expression of LPS was examined after *P. gingivalis* in was cultured in H+ medium or H− medium, as described previously (13). Briefly, late-exponential-phase cultures of *P. gingivalis* cultured in H+ media were initially transferred as a 10% (vol/vol) inoculum to anaerobically equilibrated H− media and incubated at 37°C in an anaerobic atmosphere for 24 h. The 24-h *P. gingivalis* culture in H− medium served as the

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inoculum to H+ or H− medium. Growth was monitored by A_{660} (Pharmacia LKB spectrophotometer) and by total viable cell counts determined by serial dilution on anaerobic blood agar plates. Late-exponential-phase cultures in either H+ or H− medium were used for LPS extraction. H+ medium contained 1.5 μM hemin and consisted of either a complex, commercially available medium (2.8% Schaedler broth; Difco, Detroit, Mich.) or a less complex, basal medium (14). H− medium contained no added hemin and consisted of either a complex, commercially available medium (Trypticase soy broth; Difco) or basal medium without added hemin. Basal medium contains the following per liter: trypticase peptone, 10 g; tryptophan, 0.2 g; sodium sulfite, 0.1 g; and cysteine (14).

LPS purification. LPS cultured in H+ medium (H+LPS) or H− medium (H−LPS) were extracted from *P. gingivalis* by the hot-phenol-water technique (32) followed by cesium chloride gradient centrifugation (9). Briefly, approximately 10 g (wet weight) of *P. gingivalis* was suspended in 35 ml of pyrogen-free water–35 ml of 90% phenol at 65°C added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 × *g* for 20 min and collected. This process was repeated, and the aqueous phase was pooled and dialyzed extensively against deionized water for 3 days. The dialyzed LPS preparation was subjected to cesium chloride isopycnic density gradient centrifugation (in 0.5837 g of $CsCl_2$–4.4 ml of the LPS preparation) at 42,000 rpm for 72 h in a Beckman (Palo Alto, Calif.) L-60 Ultracentrifuge. The refractive indices of the gradient fractions were determined with a refractometer, and values were converted to density (grams per milliliter). Fractions containing LPS (density fractions between 1.42 and 1.52 g/ml) were pooled, dialyzed against distilled water for 3 days, lyophilized, and stored at room temperature. LPS was analyzed for protein by the Pierce BCA protein assay. LPS samples were also separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for protein with Coomassie blue. Selected LPS samples were also subjected to proteinase K digestion, as described previously (4). Briefly, LPS samples were incubated with proteinase K (Sigma Chemical Co., St. Louis, Mo.) (100 μg per mg of LPS) for 1 h at 60°C. SDS-PAGE sample buffer was added directly to this preparation, which was heated at 100°C for 5 min and applied to the gel (data not shown).

SDS-PAGE analysis of LPS. H+LPS and H−LPS were subjected to SDS-PAGE with the Laemmli buffer system (19) using 12.5% gels or commercially prepared 10 to 20% gradient gels (Integrated Separation Systems, Hyde Park, Mass.). LPS samples suspended in saline were sonicated for 5 s and heated in SDS sample buffer at 100°C for 5 min. Gel electrophoresis was carried out at 27 mA per gel through the 4% stacking gel and 33 mA per gel through the resolving gel. The current was stopped when the dye front reached the bottom of the gel. One gel was stained with 0.2% (wt/vol) Coomassie brilliant blue R250 (Fisher Scientific Company, Fairlawn, N.J.) in 25% (vol/vol) isopropanol–7% (vol/vol) acetic acid, and the other gel was silver stained by using the Tsai and Frasch technique (29).

Electroblotting and probing nitrocellulose. Electroblotting was performed by using the buffer system of Towbin et al. (27) in the Protean 1 transfer cell (Bio-Rad, Richmond, Calif.) running at 40 V for 16 h and then 80 V for 30 minutes. Briefly, blotted nitrocellulose was cut into 5 mm strips (see Fig. 3), and the strips were placed in separate wells in an incubation tray (Bio-Rad). After being washed three times in washing buffer–Tween 20 (WBT), unreacted sites were blocked with 100 μg of human serum albumin (Sigma) in WBT, washed three times in WBT, and treated with a 1:50 dilution of patient serum in WBT overnight (or a 1:200 dilution of polyclonal rabbit antiserum [see Fig. 2]) at 4°C. After being washed three times in WBT, blots were probed with a 1:2,000 dilution of rabbit anti-human immunoglobulin G horseradish peroxidase (or goat anti-rabbit immunoglobulin G-horseradish peroxidase for Fig. 2) for 30 min. After being washed three times in WBT and once in washing buffer, bands were developed with 0.06% 4-chloro-1-naphthol in washing buffer and 30% H_2O_2. The color reaction was stopped with washing buffer at the desired intensity.

Generation of rabbit antiserum. Antisera to *P. gingivalis* strains grown in H+ medium (Schaedler broth) were produced in New Zealand White female rabbits (3 to 4 kg) with the use of a procedure described by Parent et al. (23). Briefly, the rabbits received daily intravenous injections into the marginal ear vein with increasing doses of a formalin-killed bacterial suspension (approximately 1.2×10^9 cells per ml in saline) ranging from 0.3 to 1.5 ml for a total of 10 injections, and 1 week later they received a booster series of three daily injections with 1.5 ml of the antigen suspension. On day 27, 50 ml of blood was drawn from the central artery of the ear. A series of booster injections were repeated at biweekly intervals after this test bleeding, and peak antibody titers were detected by enzyme-linked immunosorbent assay (10). The sera obtained were pooled to prepare antisera against the bacteria and frozen at −70°C.

Patient selection. Sera were obtained from 24 patients with chronic adult periodontitis (AP) and 25 healthy control subjects. The minimum clinical criteria for acceptance into the study as an AP patient were (i) no previous periodontal treatment, (ii) age of at least 30 years, and (iii) one or more periodontal pockets of at least 6 mm and at least 30% bone loss at those sites. AP patient sera were further segregated by patient age, sex, race, and disease severity. Five sera were obtained from patients with mild AP (American Academy of Periodontology [AAP] type II), 12 sera were from patients with moderate AP (AAP type III), and 7 sera were from patients with severe AP (AAP type IV). The guidelines for these clinical criteria are described elsewhere (1). The 25 control patients were age, sex, and race matched and had no periodontal pockets greater than 4 mm and no detectable generalized or localized bone loss. Subjects with a history of systemic disease or receiving antimicrobial therapy or other chemotherapeutic agents 4 weeks prior to participation in the study were not included. Approximately 30 ml of blood was drawn from the antecubital vein of each patient, 20 ml was allowed to clot, and the serum was drawn off and centrifuged to remove erythrocytes. Serum was aliquoted and frozen at −70°C. A complete battery of clinical laboratory tests, including SMA-22 and complete blood count, was performed on the remainder of the blood, and patients with significant deviations from the norm were excluded from the study and advised to seek medical consultations.

Hemin binding assays. The binding of hemin to whole cells and LPS of *P. gingivalis* was analyzed spectrophotometrically, as previously described (14, 15). Whole-cell hemin binding was analyzed as follows. *P. gingivalis* grown in either H+ or H− medium (basal medium) for 24 h was pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS to an optical density at 660 nm of 1.5 (10^{10} CFU/ml). To this was added hemin to a final concentration of 30 μg/ml (based on previous saturation studies [14]). A 1.0-ml sample was immediately removed, cells were pelleted, and the supernatant was analyzed spectrophotometrically for hemin (optical density at 400 nm). The remaining cells were incubated at 37°C under anaerobic conditions and assayed at 5- to 15-min intervals for residual hemin in the supernatant. *P. gingivalis* cultures without added hemin or hemin without added cells served as a control. The concentration of hemin present in the supernatant fractions was calculated from a hemin standard curve. Binding of hemin to whole cells was calculated as the difference between the total hemin added and the amount remaining in the supernatant at each time point compared with that at time zero (14). The binding of hemin to LPS was analyzed as follows: lyophilized H+LPS and H−LPS were reconstituted to 1 mg/ml in 50 mM Tris-HCl, pH 7.2. A 0.05-ml sample of each LPS was added to 0.60 ml of PBS containing a total of 30 μg of hemin, and the mixture was incubated for 4 h at 4°C with shaking. LPS-hemin complexes were removed by high-speed centrifugation (30,000 × *g* for 60 min) followed by ultrafiltration (membrane with an M_r cutoff of 10,000) (15), and the resulting supernatant fractions were assayed spectrophotometrically for residual hemin (optical density at 400 nm). Hemin binding was calculated as described above.

RESULTS

SDS-PAGE analysis of LPS from *P. gingivalis* cultured in H+ and H− media. LPS from all *P. gingivalis* strains tested (except strain W50 BE1) separated into stepladder-like bands that migrated at a molecular mass range (relative to the migration of protein standards) of ca. 14 to 50 kDa. This migration pattern was similar to that obtained with commercially prepared smooth-chemotype LPS from *Escherichia coli* O111:B4 (Sigma Chemical Co.). Interestingly, growth of *P. gingivalis* in H+ medium (Fig. 1) resulted in a shift in the electrophoretic mobility pattern of the LPS, leading to the expression of a greater number of low-molecular-mass bands and the addition of a band at ca. 26 kDa, which we have designated the 26 LPSC. The 26 LPSC was observed for *P. gingivalis* grown in either complex (Fig. 1A) or basal (Fig. 1B) H+ medium. The 26 LPSC was refractory to Coomassie blue staining and proteinase K digestion (not shown). Interestingly, the 26 LPSC was lost, along with several other bands at <26 kDa after passage of *P. gingivalis* in H− medium, either complex (Fig. 1A) or basal (Fig. 1B). The 26 LPSC was not observed in H+LPS or H−LPS from strain W50/BE1 (Fig. 1B). The LPS from W50/BE1 migrated like a rough-chemotype LPS, similarly to that of *Salmonella typhimurium* Rc, which is a complete core polysaccharide but lacks O-specific antigen (17).

Western blot (immunoblot) analysis of H+LPS from *P. gingivalis* W83, A7436, and HG405. To determine if the hemin-induced 26 LPSC was immunogenic, antiserum was raised to strain A7436, W83, or HG405 cultured in H+ medium (Schaedler broth). As shown in Fig. 2, antisera raised to all three strains recognized the 26 LPSC in H+LPS from all of three strains but not H−LPS (Fig. 2, lanes 1 and 2). Moreover, antibodies raised to whole-cell antigens of *P. gingivalis* strains A7436, W83, or HG405 cultured in H− media did not recognize the 26 LPSC in H+LPS from strain A7436, W83, or HG405 (data not shown).

Western blot analysis of human serum reactivity to H+LPS from *P. gingivalis* A7436. Sera from patients with AP and from

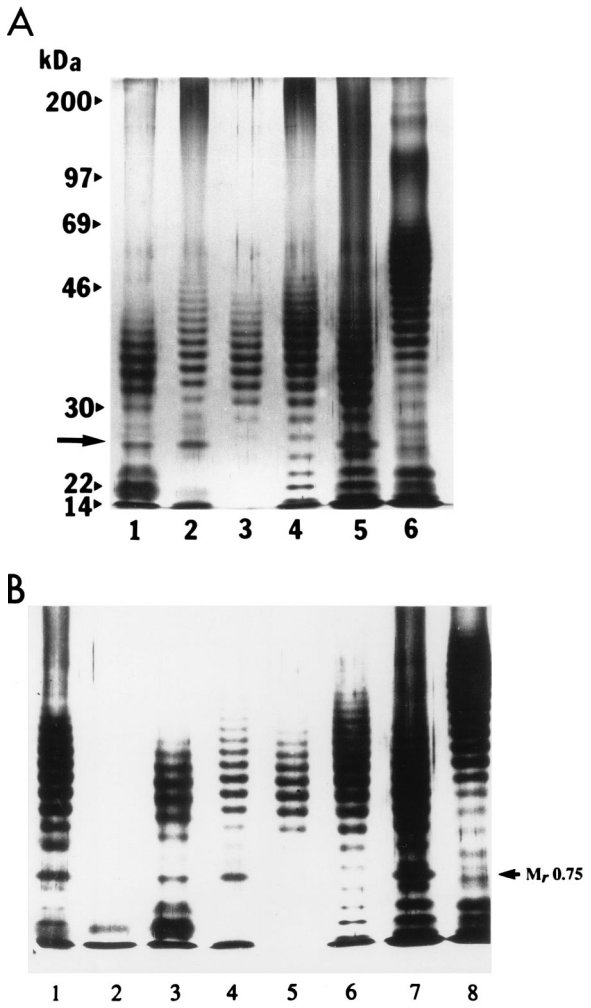

FIG. 1. SDS-PAGE analysis of H+LPS and H−LPS from *P. gingivalis*. (A) H+LPS and H−LPS purified from *P. gingivalis* cultured in complex media (see Materials and Methods). Lanes 1, 2, and 5, H+LPS from strains 33277, 381, and A7436, respectively; lanes 4 and 3, H−LPS from strain A7436 passaged once or three times, respectively, in H− medium; lane 6, LPS from *E. coli* O111:B4 (Sigma Chemical Co.). (B) H+LPS and H−LPS purified from *P. gingivalis* cultured in basal media. Lanes 1 to 4 and 7, and H+LPS from strains W50, W50/BE1, 33277, HG864, and A7436, respectively; lanes 6 and 5, H−LPS from A7436 passaged once or three times, respectively, in H− medium; lane 8, commercially prepared *E. coli* O111:B4 LPS (Sigma Chemical Co.). The polyacrylamide concentration in the resolving gels was 12.5%. Approximately 5 μg of LPS was applied to each lane. Each gel was initially stained with Coomassie blue to detect proteins, destained, and stained by the periodic acid silver stain technique of Tsai and Frasch (29).

healthy controls were reacted with nitrocellulose strips containing Western-blotted *P. gingivalis* H+LPS. Periodontitis sera reacted with a maximum of 28 bands in the H+LPS, many of which may represent repeating subunits of the O antigen (17). These bands migrated with relative molecular masses of approximately 14 to 50 kDa (Fig. 3A). The reactivity pattern achieved with serum pooled from the 24 AP patients is shown schematically in Fig. 3C. Most of the variability in reactivity patterns, however, was observed towards the lower-molecular-mass moieties (<27 kDa), such as the 26 LPSC. For example, patient 4 serum reacted with the hemin-induced 26 LPSC (band A in Fig. 3C), as well as a band at approximately 14 kDa (band C in Fig. 3C) and a band at approximately 11 kDa (band D in Fig. 3C). Patient 7 showed very intense reactivity with the 26 LPSC and faint reactivity with band D. Patient 17 showed little reactivity to anything but the 26 LPSC and band B (approximately 20 kDa). Patient 25 reacted strongly with the 26 LPSC and band C but not band B or D. The percentage of patient or control sera of the total number of patient or control sera tested that reacted with each band is shown in Fig. 4. Over 40% of the periodontitis sera reacted with the 26 LPSC (band A), while none of the control sera reacted with the hemin-induced band. In general, 75% of periodontitis patient sera reacted strongly with multiple LPS bands, especially in the range of 27 to 34 kDa. Only 20% of C sera reacted with more than one LPS band, and most reactivity was also in the range of 27 to 34 kDa. In contrast, blotting with LPS from *E. coli* O55:B5 revealed no discernible differences in periodontitis and control serum reactivity (not shown).

A cluster analysis (BMDP [Menlo Park, Calif.] statistical package) was performed to determine the association between reactivity to LPS and the age, sex, race, and disease status of the patients and controls (not shown). The analysis revealed that 75% of the 24 AP patient sera that reacted with the 26 LPSC clustered together and were drawn from patients diagnosed with moderate to severe periodontitis. In contrast, the type II or mild-periodontitis patients reacted with fewer LPS bands and clustered in the middle. The 26 LPSC was recognized by only 20% of the five type II patient sera. The controls, with few exceptions, clustered at the bottom and were not reactive with the 26 LPSC. No clustering was found for age, sex, or race.

Hemin binding by *P. gingivalis* whole cells and LPS. As shown in Table 1, H+LPS (or H+ whole cells) from *P. gingivalis* A7436 bound significantly more hemin than H−LPS (or H− whole cells). In contrast, H+LPS from strain W50/BEI did not bind hemin. There was no difference in hemin binding between H+LPS and H−LPS from the transpositional insertional mutant strain MSM-3, which has a defect in the transport and utilization of heme (13).

DISCUSSION

The results of the present study suggest that growth of *P. gingivalis* in H+ media can lead to the expression of additional antigenic determinants on the LPS. Similar modifications have

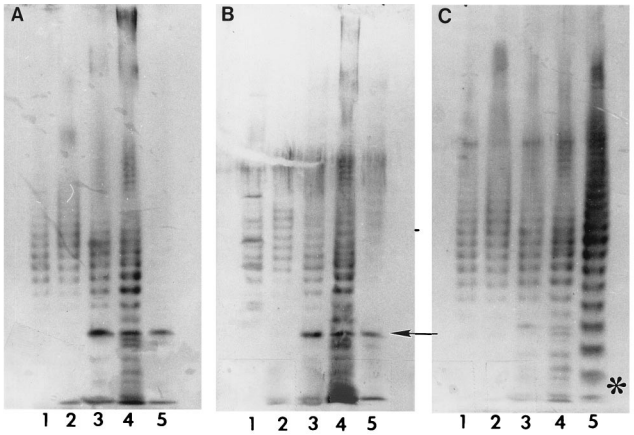

FIG. 2. Western blot analysis of H+LPS and H−LPS from *P. gingivalis* A7436, W83, and HG405. A 1:200 (vol/vol) dilution of rabbit anti-A7436 (A), anti-W83 (B), or anti-HG405 (C) was used. Lanes 1, H−LPS from strain A7436 (passaged once in H− medium); lanes 2, H−LPS from strain A7436 (passaged twice in H− medium); lanes 3, H+LPS from A4736; lanes 4, H+LPS from strain W83; lanes 5, H+LPS from strain HG405. Arrow, 26 LPSC.

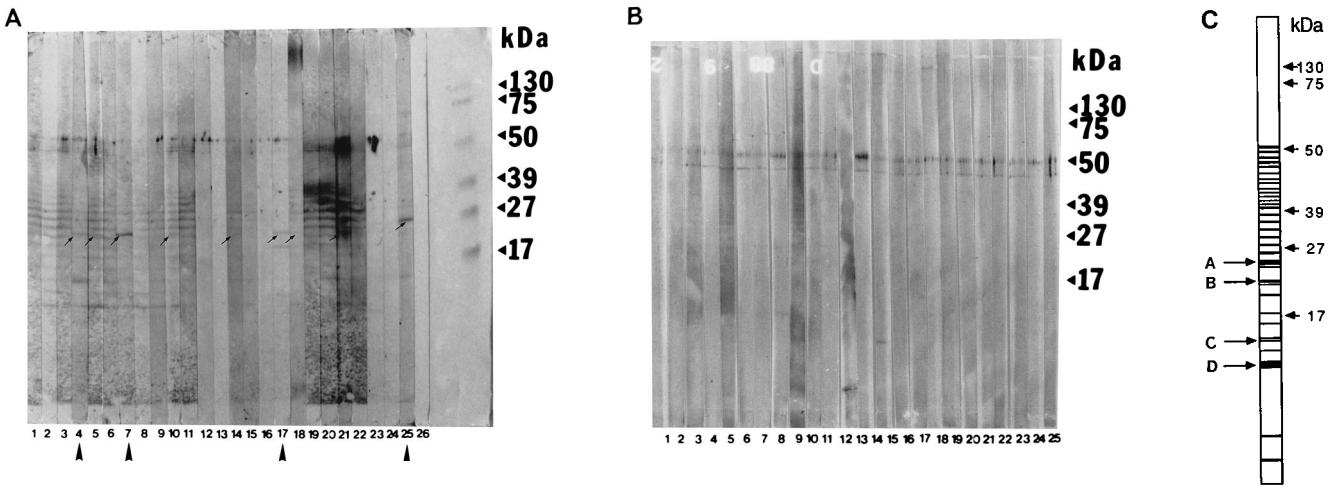

FIG. 3. Human serum reactivity with H+LPS from strain A7436. A gel was Western blotted to two nitrocellulose sheets, which were cut into strips and reacted with 1:50 (vol/vol) dilutions of 24 individual AP patient sera (A) or healthy control patient sera (B). (C) Reactivity pattern achieved with serum pooled from all 24 AP patients. Lane 26, control serum (lane 25 in panel A). The results shown are representative of a minimum of five separate analyses performed with the same LPS and the same sera. The gels were 10 to 20% commercially obtained gradient gels (Integrated Separation Systems, Hyde Park, Mass.).

been observed in the LPS of a number of human pathogens subjected to variations in environmental stresses including CO_2 and O_2 concentration (8), glucose concentration (31), temperature (18), medium aeration (28), and growth in different body fluids (5). The latter study, involving *Klebsiella pneumonia*, may be particularly relevant to our findings. A similar shift in the LPS profile, involving the low-molecular-mass antigens, was observed after growth of *K. pneumonia* in peritoneal dialysate or serum; however, the hemin or iron concentration in these body fluids was not specified. Hemin has been shown to regulate the expression of many virulence factors of *P. gingivalis*, including outer membrane proteins, fimbriae, outer membrane vesicles, hemolytic activity, protease activity, collagenolytic activity, and hemagglutination (for a review, see reference 13); however, this is the first report of a hemin-induced structural modification of LPS.

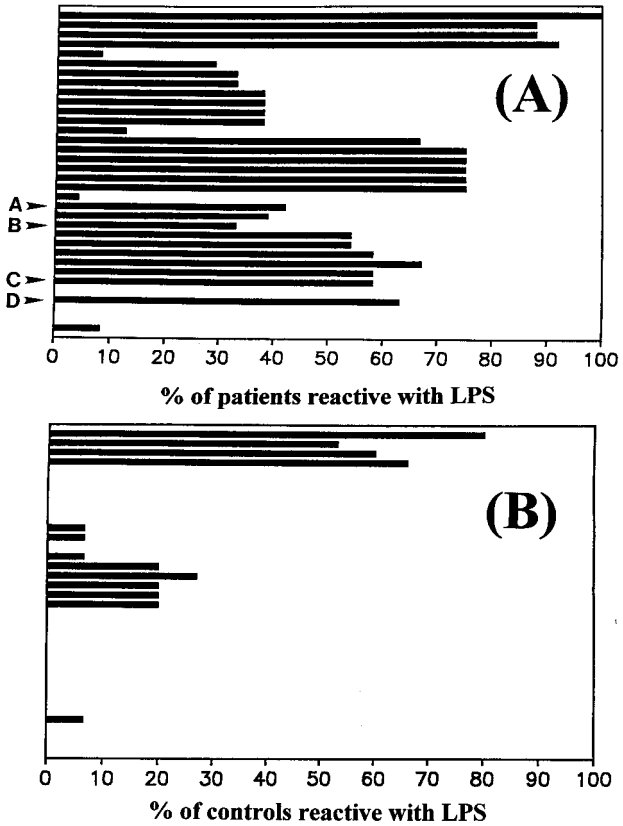

FIG. 4. Percentages of periodontitis patient sera (A) and healthy control sera (B) that reacted in a Western blot with 28 H+LPS bands from *P. gingivalis* A7436. Bands A to D (Fig. 3C), bands that migrated at approximately 26 (26 LPSC), 20, 14, and 11 kDa, respectively.

These observations may have particular relevance to periodontal disease and other chronic inflammatory diseases that are characterized by epithelial cell ulceration, increased vascular permeability (11, 22, 25), and leakage of blood into the tissues (13). The modulation of LPS by hemin is apparently functional (6) as well as structural. Champagne et al. (6) have shown that H+LPS from *P. gingivalis* (which they have termed "hemin-normal LPS") is a more potent agonist for polymorphonuclear leukocyte superoxide release than non-hemin-modulated LPS. Polymorphonuclear leukocytes are one of the

TABLE 1. Hemin binding by *P. gingivalis* LPS and whole cells

Sample and medium	Hemin bound[a]
A7436 whole cells	
H−	1.490 ± 0.05
H+	2.620 ± 0.04**
A7436 LPS	
H−	1.027 ± 0.14
H+	2.041 ± 0.10**
MSM-3 LPS	
H−	3.263 ± 0.6
H+	3.835 ± 0.5
W50 LPS, H+	1.095[b]
W50/BEI LPS, H+	0[b]
33277 LPS, H+	0.375[b]

[a] Mean of three separate determinations ± standard deviation in spectrophotometric units, calculated as described in Materials and Methods. **, statistically significant increase, as determined by Student's *t* test ($P < 0.005$).
[b] Average for two experiments.

first immune cell types to encounter bacterial pathogens at the site of periodontal infection (7). Polymorphonuclear leukocytes are thought to play both a protective role and a destructive role in the tissues, depending upon the activation state of the cells (7). Therefore H+LPS, if it exists in nature, may accelerate tissue destruction by activating polymorphonuclear leukocytes and other inflammatory cells to release potentially destructive reactive oxygen products, cytokines, and proteases (20). It is tempting to speculate that, as antibodies reactive with the 26 LPSC are observed in patients with periodontitis, H+LPS may exist in nature; moreover, despite the hydrophobic and membrane-constitutive nature of lipid A, it is exposed on the bacterial cell surface, predominantly on outer membrane vesicles or blebs (2). Understanding the mechanisms of enhanced activation of inflammatory cells by H+LPS may be a critical step in disease intervention.

We initially suspected that the hemin-dependent 26-kDa LPS moiety (26 LPSC) was a protein that copurified with LPS, such as the 26-kDa outer membrane hemin-binding protein described by Bramanti and Holt (3). However, the 26 LPSC proved to be refractory to Coomassie blue staining and insensitive to proteinase K digestion. Although the kinetics of growth in the H+ media was more rapid than that in the H− media (14), the induction of the 26-kDa moiety does not appear to be related to growth phase, since all cultures were harvested at late log phase of growth. In addition, we have not previously observed these LPS changes in other growth media that resulted in similar kinetics of growth (not shown). At present, the identity of the 26 LPSC is unknown, but endotoxin-associated proteins, such as porins, can be highly resistant to organic solvents and enzymes (12). Efforts are under way in our laboratories to biochemically and structurally characterize the hemin-dependent changes in LPS.

Studies by several investigators suggest that the LPS of *P. gingivalis* may play a role in sequestering hemin, which it then transports into the cell to obtain iron for growth (13). Grenier (15) has shown that *P. gingivalis* LPS binds hemin with high affinity via the lipid A moiety; moreover, Smalley et al. have suggested that LPS may serve as a storage depot for hemin (26). Genco et al. (14) have shown that growth in HRM+ media induces an increase in the binding of hemin by *P. gingivalis*. The present study indicates that H+LPS binds more hemin than H−LPS. Interestingly, H+LPS from the transpositional insertional mutant strain MSM-3, which has a defect in the utilization and transport of heme, did not bind more hemin than H−LPS. Whether the 26 LPSC plays a role in hemin binding is subject to speculation. Our finding that the LPS of the nonpigmented strain W50/BE1 does not bind hemin and does not produce the 26 LPSC in H+ media seems to support this speculation.

In summary, our data indicate that growth of *P. gingivalis* in H+ media resulted in additional antigenic determinants in the LPS, including a 26-kDa LPS moiety (26 LPSC), as well as an increased capacity to bind hemin. These changes in LPS were reversed when the bacteria were transferred into H− media. The pleiotropic mutant strain W50/BE1, which is nonpigmented and does not agglutinate erythrocytes, lacks the 26 LPSC and does not bind hemin. The 26 LPSC is nonproteinaceous, immunogenic, and antigenically similar among three *P. gingivalis* strains tested. A cluster analysis revealed that immunoreactivity to the 26 LPSC, as well as other low-molecular-mass LPS components, clustered with the periodontal disease status of the patients.

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