Antibody Reactive With *Porphyromonas gingivalis* Serotypes K1-6 in Adult and Generalized Early-Onset Periodontitis

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Background: Six serotypes of *Porphyromonas gingivalis* have recently been described. We sought to test the hypothesis that serotype specific carbohydrates from these strains are important antigens that elicit potent immune responses.

Methods: Serum concentrations of IgG reactive with *P. gingivalis* serotypes K1-K6 were determined for 28 adult (AP) and 28 generalized early-onset (G-EOP) periodontitis patients previously determined to be seropositive for a broken cell preparation of *P. gingivalis*. To confirm relationships suggested for K1, K2, and K6 in the analysis of initial data, the study population was increased to 133.

Results: Frequency of seropositivity for the 6 serotypes ranged from 26 to 54% of subjects. IgG concentrations ranged from 0 to 453 µg/ml with many subjects seropositive to more than one serotype. Concentrations for the subset of patients who was seropositive were high (mean responses ranged from 20 to 105 µg/ml for the 6 serotypes). Significant correlations between seropositivity to serotypes K1 and K5 as well as between K5 and K6 were found.

Conclusions: We examined the relationship of diagnosis, race, gender, smoking, probing depth, attachment loss, and antibody reaction with the *P. gingivalis* serotypes by analysis of variance. Initial findings suggested potential relationships between diagnosis, smoking, race, gender, and antibody reactive with serotypes K1, K2, and K6. A significant relationship did exist between smoking and decreased antibody reactive with *P. gingivalis* serotype K2. No other relationships were substantiated. We also examined the IgG subclass distribution and found that responses were almost exclusively IgG2. These data support the concept that antibody responses to all 6 serotypes are common in both AP and G-EOP and that these K serotype carbohydrates elicit potent IgG2 responses. *J Periodontol* 1999; 70: 730-735.

**KEY WORDS**

Antibodies; antigens, K; IgG; periodontitis; serotype; *Porphyromonas gingivalis*.

* P. gingivalis is a microorganism frequently found in periodontitis lesions.¹-⁵ Data also indicate that it is a significant risk factor for periodontitis.⁶ We recently examined IgG antibody reactive with *P. gingivalis* and found that 30 to 45% of adult and early-onset periodontitis patients were seropositive, while only 5% of healthy controls were seropositive.⁷ Interestingly, antibody reactive with *P. gingivalis* is associated with decreased attachment loss in early-onset periodontitis suggesting it may play an important role in host defense.⁸ Laine et al.⁹ and van Winkelhoff et al.¹⁰ have recently defined 6 *P. gingivalis* serotypes (K1-K6) by use of rabbit antisera. They identified the serotype-defining antigens as capsular polysaccharide.⁹,¹⁰ Subsequently, they found the prevalence of the *P. gingivalis* K serotypes ranged from 1.1 to 23% in periodontitis patients.¹¹ Studies with *Actinobacillus actinomycetemcomitans* indicate that it is important to define the serotype when studying immune responses in a given individual as the serotype specific carbohydrate is the dominant antigen.¹²,¹³ Further, the response to this antigen is affected by smoking, associated with better periodontal health, related to diagnosis, and is higher in black than in white subjects.¹²-¹⁷

The objectives of this study were to determine if the serotype specific antigens elicit potent immune responses and to examine the relationship of IgG concentrations reactive with the 6 *P. gingivalis* K
serotype-defining antigens in adult (AP) and generalized early-onset periodontitis (G-EOP) patients. The patients were seropositive (≥2 µg/ml antibody) for a broken cell preparation of P. gingivalis strain W83. The relationship between reactivity with the P. gingivalis serotypes and diagnosis, race, gender, smoking, probing depth, attachment loss, and IgG subclass was examined. The results indicated that all 6 serotypes elicited IgG2 responses in both AP and G-EOP and smoking can be an important factor in this response.

MATERIALS AND METHODS

Study Population
All human subjects had a clinical periodontal examination and provided demographic information (Table I) as well as a blood sample. Subjects were all seropositive for a P. gingivalis strain W83 broken cell preparation (≥ 2 µg/ml antibody). These patients were classified as follows:

Adult periodontitis. chronic periodontitis generalized to all 4 quadrants, although cuspids and incisors involvement may be absent. Subjects had 3 of 4 of their first molars with probing depths (PD) in interproximal sites of 5 to 7 mm with approximately the same loss of attachment. Subjects ranged in age from 35 to 55 years of age.

Generalized early-onset periodontitis. 35 years or less with a generalized pattern of severe destruction, with attachment loss of at least 5 mm on 8 or more teeth, at least 3 of which were not first molars or incisors.

Bacterial Strains and Growth Conditions
Porphyromonas gingivalis strains W50 (K1), HG 184 (K2), A7A1-28 (K3), ATCC 49417 (K4), HG 1690 (K5), and HG 1691 (K6) were employed in this study. Bacteria were grown in brain heart infusion (BHI) broth,§ supplemented with hemin,§ (5 mg/liter) and vitamin K1§ (1 µl/liter) as previously described.18

Purification of Polysaccharide
Phenol-water extracts were prepared and subjected to gel filtration chromatography using modifications of a previously described method.18 The materials were dissolved in 1.5% deoxycholate§ (DOC), 50 mM Tris-HCl, 1 mM EDTA, pH 9.5, passed through a syringe filter 110 µm (0.2 µm), applied to a column of Sephacryl S-400 HR§ (2.5 x 40 cm), and eluted with the 1.5% DOC buffer at room temperature. Fractions of 10 ml were collected and analyzed for ultraviolet absorptive materials at 280 nm, for LPS by SDS-PAGE using a silver stain, and for immunologically reactive material by double immunodiffusion in 1% agarose.* Fractions containing either polysaccharide or LPS were pooled, precipitated with 5 volumes of 95% ethanol, isolated by centrifugation, dialyzed against distilled water, and lyophilized.

Enzyme-Linked Immunosorbent Assay (ELISA)
Serum IgG antibodies to P. gingivalis K antigens were quantified by ELISA. Briefly, 100 ml of 0.015 M carbonate, pH 9.6, containing 1 µg/ml of K antigen preparation, were transferred to wells of a 96-well microtiter plate.** The plates were then incubated at 4°C for 16 hours and then washed in tap water 20 times. Sera were then serially diluted (from 1:50 to 1:102,400) in diluent (equal volumes of distilled water containing 10% nonfat dry milk and PBS, pH 7.2). Each dilution of serum was added to 2 antigen-coated wells and 2 uncoated wells. The final quantity of serum in the well varied from 1/250 ml in the first well to 1/512,000 ml in the final well. The uncoated wells served as a background control. After incubation and washing as described above, 100 microliters of a 1:1000 dilution of alkaline phosphatase labeled goat anti-human IgG (heavy and light chain specific)†† or a 1:1000 dilution of mouse anti-IgG1-4 monoclonal antibodies‡‡ followed by a 1:1000 dilution of alkaline phosphatase labeled goat anti-mouse IgG (heavy and light chain specific)†† in diluent was added per well. After incubation for 16 hours and washing, 100 microliters of substrate (p-nitrophenylphosphate 1 mg/ml in diethanolamine buffer) was added and incubated for 30 minutes at room temperature. The optical density (O.D.) at 405 nm for each well was then measured using a Vmax microplate reader.§§ The difference in O.D. (delta optical density between coated and uncoated wells) was the basic data unit.

Table I. Demographic Data on Adult and Generalized Early-Onset Periodontitis Patients

<table>
<thead>
<tr>
<th>Race</th>
<th>Diagnosis</th>
<th>N</th>
<th>Age (mean±SE)</th>
<th>Probing Depth (mean±SE)</th>
<th>Attachment Loss (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>AP</td>
<td>50</td>
<td>42±2</td>
<td>2.81±0.08</td>
<td>1.87±0.21</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>46</td>
<td>27±1</td>
<td>4.01±0.16</td>
<td>2.89±0.28</td>
</tr>
<tr>
<td>White</td>
<td>AP</td>
<td>14</td>
<td>47±3</td>
<td>2.82±0.21</td>
<td>1.92±0.39</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>23</td>
<td>29±1</td>
<td>3.34±0.13</td>
<td>2.69±0.25</td>
</tr>
</tbody>
</table>

§ Sigma Chemical Co., St. Louis, MO.
¶ Acrodisc PF, Gelman Sciences, Ann Arbor, MI.
ǁ Pharmacia Fine Chemicals, Piscataway, NJ.
‡ J.T. Baker, Inc., Phillipsburg, NJ.
†† Immulon 1, Dynatech Laboratories, Inc., Alexandria, VA.
‡‡ Kiregard and Perry Laboratories, Inc., Gaithersburg, MD.
§§ Molecular Devices, Sunnyvale, CA.
Optical density readings were converted to an equivalent concentration of IgG antibodies by means of heterologous interpolation, as previously described. Briefly, a standard curve was constructed using α-NPBSA human-mouse chimeric antibodies and NP-BSA as the antigen. A plot of OD readings versus α-NPBSA mouse/human IgG2 chimera was then utilized to estimate IgG concentration in patient sera using the corresponding OD values. A patient was considered seropositive if any antibody was detectable. Selectivity and specificity was demonstrated in this assay as at most 54% of subjects responded to any given antigen and with the exception of 1 individual, subjects did not respond to all 6 antigens.

Radioimmunoassay for Detection of Serum Cotinine
Use and/or exposure to tobacco products in our study population was assessed by means of a radioimmunoassay for cotinine, a nicotine metabolite. This analysis was performed using a commercially available nicotine metabolite kit according to the manufacturer’s instructions, and is intended for use as an aid in differentiating smokers from non-smokers. Employing this assay, we determined that patients with serum cotinine concentrations >50 ng/ml may be considered smokers.

Statistical Methods
Correlations between antibody responses to the 6 antigens were evaluated with Spearman correlation coefficient. The acceptable level of significance was 0.01. In the investigation of relationships between antibody responses and demographic and clinical variables, stepwise linear regression was used with a significance level of 0.01. The comparisons were not corrected for the multiple variables involved. Due to the lack of correction and the number of variables being investigated, we were concerned that any relationships between demographic and clinical variables and antibody levels reactive with these antigens may be spurious. For this reason we obtained an additional sample of subjects to verify any significant relationships.

RESULTS
Immunoglobulin G antibody concentrations reactive with the serotype defining capsular polysaccharide antigens for *P. gingivalis* serotypes K1-K6 were determined for AP and G-EOP patients previously found to be seropositive (≥2 µg/ml of antibody reactive with *P. gingivalis* broken cell preparations)(Table 2). The range of the responses was 0 to 453 µg/ml with many patients seropositive for more than one strain. The percent of subjects that were seropositive for each of the K serotypes was: K1 = 39%, K2 = 40%, K3 = 49%, K4 = 26%, K5 = 28%, and K6 = 54%, indicating that each of the K serotypes was well represented in this group of patients. The mean response of all subjects and the seropositive subset of subjects for each antigen is listed in Table 2. It should be noted that the serum levels were very high in the seropositive subjects with averages ranging from 20 to 105 µg/ml. The Spearman rank correlations between antibody responses to the 6 serotypes are summarized in Table 3. Significant positive correlations existed between serotypes for K1 and K5 and for K5 and K6. For serotypes K5 and K6, 93% of the subjects that were seropositive for K5 were also seropositive for K6, while only 48% of the subjects seropositive for K6 were also seropositive for K5. The K1-K5 relationship was more symmetric with 9/57 seropositive for both and 28/57 seronegative for both (65% agreement).

### Table 2.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Diagnosis</th>
<th>Mean Antibody Concentration (µg/ml±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>All Patients</td>
</tr>
<tr>
<td>K1</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
<tr>
<td>K2</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
<tr>
<td>K3</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
<tr>
<td>K4</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
<tr>
<td>K5</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
<tr>
<td>K6</td>
<td>AP</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>G-EOP</td>
<td>28</td>
</tr>
</tbody>
</table>

* 3/56 did not respond to any of the 6 serotypes, 1/56 responded to all 6 serotypes.
We next examined the relationships of diagnosis, race, gender, smoking, probing depth, attachment loss, and antibody reactive with the *P. gingivalis* serotypes by stepwise analysis of variance. Initial findings on 56 patients suggested potential relationships between diagnosis, smoking, race, gender, and antibody reactive with serotypes K1, K2, and K6. To confirm these relationships, the subject number was increased to 133 subjects for additional analysis of these relationships for K1, K2, and K6. As shown in Table 4, the data indicate that a significant relationship existed between smoking and decreased antibody reactive with *P. gingivalis* serotype K2. Other relationships suggested in the initial analysis did not reach statistical significance in the larger data set. We then reanalyzed the data on the effect of smoking without the black adult periodontitis patients, as previous work has shown that smoking is associated with lower total and *A. actinomycetemcomitans* specific IgG2 antibody in AP whites and G-EOP but not AP blacks or LJP patients (Table 5).

Consistent with previous results, omission of black AP subjects (which included smokers who were not significantly different from non-smokers) from the data set strengthened the association of smoking with lower levels of anti-K2. Thus, antibody reactive with the antigen is decreased by nearly 90% in smoking AP whites, and G-EOP subjects.

Carbohydrate antigens typically elicit IgG2 dominated responses as illustrated by the serotype specific antigen of *A. actinomycetemcomitans*. This prompted us to examine the IgG subclass distribution for all *P. gingivalis* K antigens. As shown in Figure 1, all of the 6 *P. gingivalis* K antigens elicited IgG2 antibody with virtually none of the other subclasses.

**DISCUSSION**

Antibody concentrations reactive with *P. gingivalis* K1-K6 serotype defining antigens were determined using a standard ELISA. Antibody reactive with all 6 serotypes was found in a percentage of AP and G-EOP patients at concentrations reaching 453 µg/ml. The average IgG concentrations for seropositive patients was high (mean responses ranged from 20 to 105 µg/ml for the 6 serotypes) and in many cases in the range found for the serotype specific antigen of *A. actinomycetemcomitans*. In addition, patients with only modest IgG reactive with a *P. gingivalis* W83 broken cell preparation were found to have much more IgG reactive with the appropriate serotype specific carbohydrate. This indicates the importance of using the appropriate serotype as the antigen in studies of antibody responses elicited by *P. gingivalis*, as is the case for *A. actinomycetemcomitans* where patients seropositive for serotype b typically have very little antibody reactive with serotypes a or c and vice versa. Significant

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**Table 3.**

**Correlation of Seropositivity Among *Porphyromonas gingivalis* K antigens K1-K6 in Adult and Generalized Early-Onset Periodontitis Patients**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>K2</th>
<th>K3</th>
<th>K4</th>
<th>K5</th>
<th>K6</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>0.21</td>
<td>0.20</td>
<td>0.25</td>
<td>0.35*</td>
<td>0.02</td>
</tr>
<tr>
<td>K2</td>
<td>0.14</td>
<td>0.19</td>
<td>0.03</td>
<td>-0.01</td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td>0.21</td>
<td></td>
<td>0.03</td>
<td>-0.26</td>
<td></td>
</tr>
<tr>
<td>K4</td>
<td></td>
<td></td>
<td>0.20</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>K5</td>
<td></td>
<td></td>
<td></td>
<td>0.47*</td>
<td></td>
</tr>
</tbody>
</table>

* *P* <0.01.

**Table 4.**

**Relationship Between Antibody Reactive With the K2 Serotype Defining Antigen and Smoking in Adult and Generalized Early-Onset Periodontitis**

<table>
<thead>
<tr>
<th>Smoker</th>
<th>N</th>
<th>Mean Antibody Concentration (µg/ml±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>85</td>
<td>31.8±8.4</td>
</tr>
<tr>
<td>Yes</td>
<td>48</td>
<td>9.0±2.2*</td>
</tr>
</tbody>
</table>

* Significant difference between groups at *P*≤0.01.

**Table 5.**

**Relationship Between Antibody Reactive With the K2 Serotype Defining Antigen and Smoking in Adult and Generalized Early-Onset Periodontitis: Influence of Black AP Patients**

<table>
<thead>
<tr>
<th>Smoker</th>
<th>N</th>
<th>Mean Antibody Concentration (µg/ml±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>White AP, G-EOP</td>
</tr>
<tr>
<td>No</td>
<td>53</td>
<td>35.6 ± 12.4</td>
</tr>
<tr>
<td>Yes</td>
<td>30</td>
<td>4.3 ± 1.0†</td>
</tr>
</tbody>
</table>

* Subjects were placed into 2 groups: one that included white AP and black and white G-EOP patients, and a second including only AP black patients. This was done since previous work has shown that smoking affects serum IgG2 for the first group of subjects but not for black AP subjects.

† Significant difference between groups at *P*≤0.0025.
correlations between antibody reactive with K1/K5 and K5/K6 were found suggesting these serotypes may be present either in combination or succession. Furthermore, smoking appears to be associated with decreased antibody reactive with serotype K2 in white AP and G-EOP as was found for *A. actinomycetemcomitans* serotype b.

Lopatin and Blackburn found that most of the IgG antibody reactive with *P. gingivalis* was IgG1 and IgG2 and that most of the IgG2 was reactive with LPS. In contrast, Whitney et al. found the predominant IgG subclasses reactive with *P. gingivalis* were IgG2 and IgG3. Chen et al. found most of the antibody reactive with *P. gingivalis* was reactive with the whole-cell protein fraction with lesser amounts of antibody reactive with lipopolysaccharide and no IgG reactive with capsular polysaccharide. We have found that antibody reactive with all 6 serotype K antigens was almost exclusively IgG2.

The smoking effect on IgG2 antibody production for serotype K2 was observed in white AP and G-EOP patients, but not black AP patients. Further, removing black patients from the analysis resulted in a stronger relationship between smoking and decreased K2 reactive IgG2. We have previously shown that smoking is associated with lower total IgG2 and IgG2 antibody reactive with *A. actinomycetemcomitans* serotype b.

Figure 1. IgG subclass levels for 6 high responding patients for Porphyromonas gingivalis K antigens K1-K6 (mean+SE).

high molecular weight lipopolysaccharide. This smoking effect on total IgG2 and the *A. actinomycetemcomitans* specific IgG2 response was true for white AP subjects and G-EOP subjects, but not localized juvenile periodontitis or black AP patients. This effect was dramatic for both *A. actinomycetemcomitans* and *P. gingivalis* serotype K2 (5- to 10-fold decrease in smokers). It is interesting that the smoking effects on IgG2 parallel the effects of smoking on attachment loss in that smoking results in increased attachment loss for white AP and G-EOP, but not black AP patients. In contrast, we did not, in previous work, find an effect of smoking on the IgG2 dominated responses of the antigens phosphorylcholine, or *Haemophilus influenzae* polysaccharide. In the current study, we did not find a smoking effect on 5 of 6 *P. gingivalis* K serotype specific carbohydrates, also IgG2 dominated responses.

In this report we, therefore, have found the second specific antigen that elicits mostly IgG2 where the antibody response is apparently influenced by smoking. Why only selected IgG2 dominated responses are not affected by smoking is not understood. The decrease in the IgG2 reactive with *P. gingivalis* serotype K2 may be important, as the decrease in IgG2 antibody reactive with *A. actinomycetemcomitans* by smoking appears to explain, in part, the deleterious effects of smoking on the periodontium. Further study may find this relationship in other periodontitis associated organisms.
In conclusion, antibody reactive with the 6 \( P. gingivalis \) serotype specific polysaccharide antigens (K1-K6) is an IgG2 response that is common to both adult and generalized early-onset periodontitis and there are significant correlations between some of the serotypes. A significant relationship exists between smoking and decreased antibody reactive with \( P. gingivalis \) serotype K2.

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**REFERENCES**


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