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Immunoglobulin Allotypes and Immunoglobulin G Subclass Responses to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in Early-Onset Periodontitis

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The present study was performed to estimate the observed frequencies of the immunoglobulin heavy-chain (Gm) and light-chain (Km) allotypes among patients with early-onset periodontitis (EOP) and their effect on the IgG2 subclass responses against *Actinobacillus actinomycetemcomitans* Y4 and *Porphyromonas gingivalis* 381, respectively. Sixty-nine EOP patients, including 11 with localized juvenile periodontitis (LJP), 19 who had LJP, 15 with LJP-rapidly progressing periodontitis (RPP), and 24 with RPP, were examined for the Gm and Km allotypes by a hemagglutination inhibition test. Levels of immunoglobulin G2 (IgG2) antibodies against the two organisms were determined by enzyme-linked immunosorbent assay. Fifty race- and age-matched, periodontally healthy subjects were also included as a control group. The observed frequencies of the Gm haplotype afnb and Km(1) were significantly higher in the RPP and LJP groups, respectively. The G2m(n)+ group of those with RPP and the Km(1)+ group of those with LJP had significantly higher levels of IgG2 antibodies to *A. actinomycetemcomitans* and *P. gingivalis*, respectively. The results indicate that linkage disequilibrium of the G2m(n) locus in RPP patients or the Km(1) locus in LJP patients may be associated with high IgG2 antibody responses to the respective bacteria. It was reasoned that the IgG2 antibody responses are associated with the immunoglobulin allotypes. The function of IgG2 antibodies in their reaction to different bacterial antigens may be interpreted as either protective or nonprotective in the two different types of EOP (i.e., LJP and RPP).

Actinobacillus actinomycetemcomitans and *Porphyromonas gingivalis* have been implicated as the primary periodontal pathogens in localized juvenile periodontitis (LJP) and rapidly progressing periodontitis (RPP), respectively (47, 54). Host responses of RPP and LJP patients towards the relevant organisms have been extensively studied (12). However, considerable variability in the humoral immune responses against these bacteria in early-onset periodontitis (EOP) has been found (13, 20, 41, 48). The two representative bacteria have frequently been found in patients with either of these two types of EOP (4, 22), which manifest unique characteristic features (6). Moreover, immunoglobulin G (IgG) antibodies to both of these organisms were elevated more often than not, regardless of the disease type. This phenomenon is more evident in family members with a high prevalence of EOP (30, 37, 39, 44, 50), suggesting a genetic predisposition of certain race or family members to EOP (18, 24, 34). Although some genetic mechanisms have been postulated in the development of EOP (33, 34), it is unclear which group of patients is more prone to certain types of EOP and what makes family members develop one or the other form.

Schenkein (33) proposed that IgG2 responses to either *A. actinomycetemcomitans* or *P. gingivalis* may be a determining factor for the development of the localized (and hence protective) or generalized form of EOP (17, 23, 24, 31, 49, 51, 52). Racial differences in terms of levels of IgG antibodies to these periodontopathic organisms have also been reported (18, 24). Such observations suggest that a genetic factor(s) is responsible for the individual variability of the IgG response to certain periodontal pathogens, therefore making certain individuals more prone to a specific form of periodontal disease. It has been postulated that IgG subclass responses are closely associated with immunoglobulin allotypes, as well as with bacterial antigenic determinants (19, 35, 36, 45). Of several immunoglobulin allotypes, two are important: heavy-chain allotype markers (Gm) and kappa light-chain allotype markers (Km) (32, 46). The allotype markers are inherited in accordance with the Mendelian codominance trait, and unique haplotype specificities exist within individuals. Both the Gm and Km allotypic markers demonstrate considerable racial differences (25, 32). Normal IgG subclass concentrations in serum (26, 36, 38, 40, 42, 43, 53), levels of antibodies to bacterial antigens (9, 19, 47), and postimmunization levels of the IgG subclass (1, 14–16) may be influenced by the Gm and/or Km allotypes.

On the basis of our previous findings (9), it appears that IgG subclass responses to the periodontal pathogen *P. gingivalis* are closely associated with the immunoglobulin heavy-chain allotypes (Gm). Moreover, patients with certain Gm allotypes seem to be more predisposed to RPP, even within the same race. The present study was performed to (i) examine frequencies of Gm and Km in EOP patients and healthy, race- and age-matched controls in Korea and (ii) evaluate the relationship, if any, between Gm and Km allotypes and IgG subclass responses to *A. actinomycetemcomitans* and *P. gingivalis*, respectively, in patients with different forms of EOP.

MATERIALS AND METHODS

Selection of patients and serum sampling. On the basis of radiographic patterns of interproximal alveolar bone loss and attachment loss, patients demonstrating various forms of EOP were reclassified into four groups, LJP (typically localized to first molars and/or incisors), post-LJP (LJP with adjacent teeth slightly involved), LJP-RPP (rapidly progressing but maintaining localized first-molar–incisor involvement), and RPP (typically, generalized involvement), based upon criteria described previously (7, 8). For race- and age-matched controls, 50

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TABLE 1. Haplotype frequencies of immunoglobulin heavy-chain allotypes (Gm) observed in EOP patients and race- and age-matched controls

Gm haplotype	Frequency in EOP subform[a]:				Frequency in controls (n = 50)
	LJP (n = 11)	Post-LJP (n = 19)	LJP-RPP (n = 15)	RPP[b] (n = 24)	
ag(1,21)	0.5454	0.4474	0.3571	0.4271	0.5350
axg(1,2,21)	0.2273	0.2895	0.3214	0.2604	0.1950
ab3st(1,13,15,16)	0.1364	0.0526	0.1786	0.0417	0.1200
afnb1b3(1,3,5,13,23)	0.0909	0.2105	0.1429	0.2708	0.1500

[a] Subforms of EOP according to criteria of a previous study (7).
[b] All four Gm haplotypes were significantly different (either higher or lower) from the control group by chi square analysis ($P < 0.05$).

subjects without prior evidence of destructive periodontal disease were included. Patients having systemic disease which might modify the disease course or humoral immune response were excluded from the study. Peripheral blood was drawn by venipuncture, and serum samples were collected by centrifugation at 5,000 × *g* for 5 min and stored in aliquots at −20°C until used.

Preparation of bacterial antigens and measurements of IgG subclass antibody levels. Briefly, *A. actinomycetemcomitans* Y4 and *P. gingivalis* 381 were plated on tryptic soy agar-bacitracin-vancomycin (TSBV) and blood agar plates and grown in a CO_2 incubator for 3 days and in an anaerobic chamber (Coy Co.) for 7 to 10 days, respectively. The bacteria were subcultured until pure, and then colonies were grown in broth culture. After harvesting, bacterial cells were formalinized prior to use as whole-cell antigens. Serum antibody titers toward these organisms were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, 0.2 ml of formalin-fixed bacterial antigens appropriately diluted in buffer I (3.68 mM sodium carbonate, 68 mM sodium bicarbonate, 3.08 mM sodium azide) was added to each well, and the plates were incubated for 4 h at 37°C and stored overnight at 4°C. After the plates were washed three times with buffer II (84.9 mM sodium carbonate, 0.5 ml of Tween 20 per liter), a 0.1-ml serum sample diluted in buffer II was added to each well and the plates were incubated for 2 h at room temperature. The plates were washed three times with buffer II, 0.1 ml of mouse anti-human IgG2 (affinity-purified monoclonal antibody, gamma chain specific, clone SH-21; Sigma Chemicals) diluted in buffer III (19 mM dibasic sodium phosphate, 2.33 mM monobasic sodium phosphate, 149.7 mM sodium chloride, 3.08 mM sodium azide, 0.5 ml of Tween 20 per liter) was added to each well, and the plates were incubated for 2 h at room temperature. After the plates were washed three times with buffer II, 0.1 ml of goat anti-mouse IgG (heavy and light chain specific, affinity purified, alkaline phosphatase conjugated; Calbiochem, Basel, Switzerland) diluted in buffer III was added to each well and the plates were incubated overnight at room temperature. After the plates were washed, 0.2 ml of nitrophenyl phosphate (1 mg/ml) was added to each well, the plates were incubated for 30 min, and 0.1 ml of 1 N NaOH was added to stop the color reaction. Optical density was measured with an ELISA plate reader with the wavelength set at 492 nm.

To determine serum IgG antibody titers, optical densities were plotted as a function of the serum dilution factor. Regression analysis was performed, and the reciprocal of the serum dilution factor where the *x* axis intersected with an optical density of 1 was used as the ELISA unit for each sample. Antibody titers greater than twice the mean IgG titer of the control subjects were regarded as elevated.

Determination of Gm and Km immunoglobulin allotypes. The following allotype markers were identified by the hemagglutination inhibition assay in accordance with the method described by van Loghem (45), and others (1, 28): G1m(a), G1m(x), G1m(f), G2m(n), G3m(g), G3m(b) (including b0b1b3b5), G3m(s), G3m(t), Km(1), and Km(3) (Analytical Genetic Testing Center, Denver, Colo.). The Gm and Km system consisted of anti-Gm or anti-Km agglutinators and erythrocytes coated with Gm(+) or Km(+) anti-Rho antibodies (Analytical Genetic Testing Center). Anti-Rho antibodies were used as the coating antigens for erythrocytes, except for G2m(n), where erythrocytes were coated with myeloma proteins (kindly provided by H. Matsumoto, Osaka Medical College, Osaka, Japan). Test serum samples were mixed with different agglutinators and reacted with erythrocytes coated with anti-Rho antibodies. V-bottom 96-well microtiter plates were used for the assay, and the hemagglutination reaction was examined with a translucent plastic reader with the microtiter plates angled at 60° from horizontal (in accordance with the instruction manual from the Analytical Genetic Testing Center). If hemagglutination occurred, the result was recorded as negative, and if hemagglutination failed to occur, then the result was positive for the Gm or Km markers. Nine phenotypes observed among the Koreans fell into one of four Gm haplotypes. Frequency distributions of the four Gm and Km haplotypes among the four EOP subgroups were evaluated. On the basis of the measurements of IgG subclass antibody levels of patients in a previous study (18), we also correlated these patterns of elevated IgG subclass antibodies with the Gm and Km phenotypes.

Statistical analysis. A chi square test was performed to identify differences in the observed frequencies of each haplotype between the EOP subforms or adult periodontitis and the control group. The Wilcoxon signed rank-sum test was performed to identify the differences of IgG subclass antibody levels with respect to the Gm or Km allotype.

RESULTS

Gm and Km frequencies. The Gm and Km haplotype frequencies for each EOP subform are shown in Tables 1 and 2. When the Gm haplotype frequencies of EOP patients were compared with those of controls, frequencies of four haplotypes in RPP were significantly different from those of the controls, while these relationships were not observed for the other subforms (Table 1). When the Km haplotypes were compared, the Km(1) haplotype frequencies were significantly higher, while the Km(3) haplotype frequencies were significantly lower, only in LJP patients than in the control group (Table 2).

Levels of IgG2 subclass antibodies to *A. actinomycetemcomitans* and *P. gingivalis* in serum with respect to Gm and Km phenotypes. Levels of IgG2 subclass antibodies to either *A. actinomycetemcomitans* or *P. gingivalis* with respect to immunoglobulin allotypes Gm and Km in EOP patients are shown in Tables 3 and 4. The RPP G2m(n)$^+$ group and all of the EOP patients had significantly higher levels of IgG2 subclass antibodies to *P. gingivalis*, while this relationship was not observed in the LJP group or for antibodies to *A. actinomycetemcomitans* in the RPP group or all of the EOP patients (Table 3). Only the Km(1)$^+$ group of LJP patients had significantly higher levels of IgG2 antibodies to *A. actinomycetemcomitans*,

TABLE 2. Haplotype frequencies of immunoglobulin light-chain allotypes (Km) observed in EOP patients and race- and age-matched controls

Haplotype	Frequency in EOP subform[a]:				Frequency in controls (n = 50)
	LJP[b] (n = 11)	Post-LJP (n = 19)	LJP-RPP (n = 15)	RPP (n = 24)	
Km(1)	0.5910	0.3120	0.3330	0.3540	0.2650
Km(3)	0.4090	0.6880	0.6670	0.6460	0.7350

[a] Subforms of EOP according to criteria of a previous study (7).
[b] Significantly different (either higher or lower) Km haplotype frequencies from control group by chi square analysis ($P < 0.05$).

TABLE 3. Effect of G2m(n) on mean IgG2 subclass levels in LJP, RPP, and all EOP patients

Patient group and antibody subclass	Mean no. of ELISA units (no. of patients)		*P* value[a]
	G2m(n)$^+$	G2m(n)$^-$	
All EOP			
IgG2/*Pg*[b]	555.5 (23)	205.1 (46)	<0.001
IgG2/*Aa*[c]	1,897.0 (23)	1,054.0 (46)	NS[d]
LJP			
IgG2/*Pg*	527.5 (2)	177.1 (9)	NS
IgG2/*Aa*	790.0 (2)	1,073.8 (9)	NS
RPP			
IgG2/*Pg*	699.2 (14)	192.5 (10)	<0.01
IgG2/*Aa*	2,382.93 (14)	946.3 (10)	NS

[a] Determined by Wilcoxon signed rank-sum test.
[b] *Pg*, *P. gingivalis* specific.
[c] *Aa*, *A. actinomycetemcomitans* specific.
[d] NS, no significant difference.

while this relationship was not found in the other groups of patients (Table 4).

DISCUSSION

The present study was performed (i) to examine Gm and Km frequencies in EOP patients and (ii) to evaluate the effects of the Gm and/or Km allotypes on IgG subclass antibody responses to *A. actinomycetemcomitans* and *P. gingivalis*, respectively, in patients with different forms of EOP. The G2m(n) haplotype frequencies observed were significantly higher in the RPP group, while those of Km(1) were significantly higher in the LJP group, than in the control group (Table 1). Although the higher G2m(n) haplotype frequency in RPP patients was consistent with our previous report, we report here, for the first time, that the observed Km(1) frequency in LJP patients was significantly higher than that in the control group. These effects were not found in other types of EOP. Thus, the G2m(n) and Km(1) haplotypes may be closely associated with the genetic predispositions of RPP and LJP patients, respectively.

The IgG2 subclass antibody response to the bacterial antigens is associated with the immunoglobulin allotypes, i.e., Gm and Km types (1, 9, 19, 46). Our previous study demonstrated that G2m(n)$^+$ patients had higher levels of IgG2 antibodies to *P. gingivalis* than did the G2m(n)$^-$ group of EOP patients. This relationship was not demonstrated for levels of IgG2 antibodies to *A. actinomycetemcomitans* in any other types of EOP patients, except for those with RPP. Furthermore, the Km(1)$^+$ patients had significantly higher levels of IgG2 antibodies to *A. actinomycetemcomitans* in LJP, suggesting that the Km(1) locus also regulates the levels of IgG subclass antibodies to bacterial antigens in certain groups of patients (18, 24). However, this phenomenon also could not be found for *P. gingivalis* or *A. actinomycetemcomitans* in any other types of EOP patients, except for LJP patients. These findings, taken together, suggest that there might be linkage disequilibrium in the Gm or Km loci in each group (either LJP or RPP) of EOP patients with respect to specific bacterial antigens. These phenomena could well be demonstrated in the variable IgG subclass antibody responses to the immunization vaccines in racial groups with different genetic makeups. The fact that Km(1) haplotype frequencies are much higher in blacks than in whites (32), with higher levels of IgG2 antibodies to *A. actinomycetemcomitans* in black subjects, both healthy persons and LJP patients (18, 24), might explain the higher prevalence of LJP in blacks. This concept may be further applied to genetic epidemiology, although with limited comparative data; the Km(1) haplotype frequencies of blacks and whites in the United States (32) and the Koreans in the present study (Table 2) are 0.340, 0.082, and 0.265, respectively, and the reported LJP prevalences are 2.05, 0.14, and 1.12%, respectively (7, 21 [data from patients visiting outpatient clinics]).

The present observations suggest that patients positive for haplotype afnb are genetically predisposed to the development of RPP. The elevated levels of IgG2 antibodies to *P. gingivalis* might be due to linkage disequilibrium of the G2m locus in these patients. Elevated IgG2 levels may have contributed to the widespread forms of EOP. Tew et al. (41) postulated that *P. gingivalis* may be the best organism for testing the seropositivity of EOP patients. G2m(n)$^+$ patients had the highest levels of IgG2 antibodies to *P. gingivalis* of all of the EOP patients in our study. Thus, it is highly probable that EOP patients in general demonstrate high levels of IgG2 antibodies to this organism (8, 24). In our study, IgG2 levels were measured by using a commercially available murine monoclonal antibody, designated SH21 (Sigma), which is specific for the Fc portion of IgG2, where the G2m(23) allotype is located. Because this monoclonal antibody could bind preferentially to IgG2 molecules containing the G2m(23) allotype, our observation may have been influenced by the imperfect assay system. It would have been better if we had used a monoclonal antibody which is specific for the Fab region of IgG2 molecules.

The elevated IgG subclass, regulated by the Gm and/or Km allotypes, has been shown to recognize selected immunodominant antigens in a quite specific manner in bacterial infections (19), including periodontal infections (10). We have previously observed that both the IgG2 and IgG4 subclasses of antibodies to *P. gingivalis* were significantly elevated in RPP patients under the close control of G2m(n) allotypes and the IgG2 subclass recognized primarily capsular polysaccharides while the IgG4 subclass recognized primarily fimbrilin proteins of *P. gingivalis* (10). Hence, it would be interesting to evaluate how these immunodominant antigens might have played roles in the pathogenesis of RPP. Alternatively, the immunodominant antigens of *A. actinomycetemcomitans* recognized by the high IgG2 responder to *A. actinomycetemcomitans* in Km(1)$^+$ patients might have played a significant role in the pathogenic mechanisms of the development of LJP.

TABLE 4. Effect of Km(1) on mean IgG2 subclass levels in LJP, RPP, and all EOP patients

Patient group and antibody subclass	Mean no. of ELISA units (no. of patients)		*P* value[a]
	Km(1)$^+$	Km(1)$^-$	
All EOP			
IgG2/*Pg*[b]	333.1 (40)	354.8 (29)	NS[c]
IgG2/*Aa*[d]	1,427.7 (40)	1,323.5 (29)	NS
LJP			
IgG2/*Pg*	289.4 (9)	22.0 (2)	NS
IgG2/*Aa*	1,237.6 (9)	53.0 (2)	<0.05
RPP			
IgG2/*Pg*	489.9 (13)	486.0 (11)	NS
IgG2/*Aa*	1,483.5 (13)	2,137.4 (11)	NS

[a] Determined by Wilcoxon signed rank-sum test.
[b] *Pg*, *P. gingivalis* specific.
[c] NS, no significant difference.
[d] *Aa*, *A. actinomycetemcomitans* specific.

Ambrosino et al. (1) reported higher susceptibility to *Haemophilus influenzae* b infection in children without the G2m(n) allotype having lower levels of IgG2 antibodies to the encapsulated bacteria than in those who were positive for G2m(n). In contrast, G2m(n)$^+$ patients were reported to be genetically predisposed to a certain group of diseases (9, 28). One may agree that normal IgG concentrations in serum, levels of antibodies to bacterial antigens, and postimmunization IgG levels may be influenced by immunoglobulin allotypes. However, there seems to be controversy regarding the relationship between the allotypes and disease susceptibility.

The function of the IgG2 subclass antibody was considered weak because of its poor complement-fixing and opsonizing capacities (5, 35). However, some investigators have reported similar IgG1 and IgG2 antibody bactericidal activities following immunization with *H. influenzae* vaccines (2, 3). Recently, Chung et al. (11) reported that the V region repertoire of postimmunization IgG antibodies was dependent on the immunoglobulin heavy-chain haplotypes. In their study, an antibody repertoire, A2-V_kII(A2) antibodies, was found to be expressed less in patients with the fb or zag C_H haplotype, while Nahm et al. (27) reported that this antibody repertoire had higher avidities (27). Therefore, expression of a light-chain variable-region repertoire of an IgG subclass antibody should be considered when estimating its functional role. Recently, Nakashima et al. (29) reported that variable light chains of the elevated IgG2 antibodies to *A. actinomycetemcomitans* serotype b were all V_kII, suggesting that the gene coding for the IgG2 reactive to *A. actinomycetemcomitans* serotype b carbohydrate is very restricted. If we consider that the fb haplotype, in either heterozygous or homozygous form, regulating IgG2 responses to polysaccharide antigens is associated with less expression of the A2-V_kII gene (11), the linkage disequilibrium of IgG2 to *A. actinomycetemcomitans* may not be associated to the fb haplotype. Rather, it might be associated with Km(1), which might restrict the expression of a VL repertoire to A2-V_kII, demonstrating higher avidity. This may explain, in part, why IgG2 antibodies to *A. actinomycetemcomitans* may be functionally protective, limiting the disease to first molars and/or incisors in LJP (34). We observed significantly higher frequencies of Km(1) in blacks (Table 2). It is also presumable that the non-antibody-using A2-V_kII gene product, dependent on the Gm allotypes of RPP patients, is associated with the poor protective effect of elevated levels of IgG2 antibodies to *P. gingivalis* associated with the linkage disequilibrium of the allotype. We are performing experiments to examine the variable light-chain repertoire of IgG2 antibodies in RPP patients.

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