

# The Relationship Between Gingivitis and the Serum Antibodies to the Microbiota Associated With Periodontal Disease in Children With Down's Syndrome

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GINGIVAL INFLAMMATION IN DOWN'S SYNDROME children (DS) develops earlier and is more rapid and extensive than in non-DS children. Abnormalities in host response to the oral flora have been proposed as etiological factors of this gingival inflammation. However, the relationship between gingivitis and the host response to oral microorganisms in DS by age has not been determined. The objective of this study was to clarify this relationship. Sera were obtained from 75 DS subjects (aged 2 to 18 years) and their gingival health assessed using a modified PMA Index (M-PMA). Antibody titers to *Porphyromonas gingivalis* (*Pg*), *Prevotella intermedia* (*Pi*), *Treponema denticola* (*Td*), *Fusobacterium nucleatum* (*Fn*), *Selenomonas sputigena* (*Sel*), *Actinobacillus actinomycetemcomitans* (*Aa*), and *Streptococcus mitis* (*Mi*) were determined using the micro-ELISA. DS subjects under 4 years old were found to have significantly more gingival inflammation than did normal children the same age. A significant positive correlation ( $r = 0.548$ ,  $P < 0.0001$ ) existed in the relationship between M-PMA score and plaque score for subjects in the G1 age group (deciduous dentition). At G1, the average antibody titers to *Aa*, *Mi*, and *Fn* exceeded those of the normal adult reference serum pool. In addition, IgG antibody titers to *Pg*, *Aa*, *Fn*, *Sel*, and *Mi* correlated significantly with the M-PMA scores in the G1 age group. There was a correlation between age (2 to 18 years) and these antibody titers. IgG antibody titers to *Pg*, *Aa*, *Sel*, and *Mi* increased significantly with increasing M-PMA score. Furthermore, the IgG antibody titers to *Pg* were higher ( $P < 0.05$ ) in the most extensive disease group compared to the DS no-disease group. The IgG antibody titers to *Pg* at G3 (early puberty) were significantly higher when compared to G1 (preschool children). The IgM antibody titers to *Aa* at G3 were higher ( $P < 0.05$ ) when compared to G1. This study suggests that colonization by *Aa* and *Fn* are closely associated with the onset of gingival inflammation in DS patients under 5 years old. Colonization by *Pg*, *Aa*, *Sel*, and *Mi* in DS appears to be associated with gingivitis at puberty. *J Periodontol* 1997;68:626–631.

**Key Words:** Antibodies, bacterial; Down's syndrome; gingivitis/etiology; gingivitis/microbiology.

Previous investigations of periodontal disease in children with Down's syndrome described a high prevalence of rapidly progressing extensive gingivitis.<sup>1–3</sup> Gingival inflammation was reported to develop earlier and was more

rapid and extensive than in non-DS children.<sup>4</sup> Abnormalities in systemic host defense mechanisms, including polymorphonuclear leukocyte chemotaxis and phagocytosis, have been proposed to contribute to the etiology of this rapidly progressive gingival inflammation.<sup>5–7</sup>

There are several studies of the microbiota and gingival inflammation in DS children.<sup>8–11</sup> Among these, Meskin et al.<sup>8</sup> implicated *Bacteroides melaninogenicus* as a possible

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**Table 1. Number and Mean Age of Down's Syndrome Children by Hellman Dental Age (HDA) Category**

HDA Category	Sex	Dental Age	
		N	Mean
G1	Male	26	4.5
	Female	18	5.7
	Total	44	5.0
G2	Male	4	7.3
	Female	4	7.5
	Total	8	7.4
G3	Male	17	10.6
	Female	6	9.3
	Total	23	10.3

The mean age of males (n = 47) is 7.0 years; of females (n = 28), 6.7 years; of all children (n = 75), 6.9 years.

etiological agent in periodontal disease, and Barr-Agholme et al.<sup>11</sup> demonstrated an altered microbial composition of the subgingival plaque of Down's syndrome subjects compared to healthy controls, with a higher frequency of *Actinobacillus actinomycetemcomitans* in DS. However, the relationship between periodontal status, microbial colonization, and age has not been described. The purpose of this study was to establish the relationship between gingivitis and serum antibody titers to periodontal disease-associated microorganisms in children with Down's syndrome.

## MATERIALS AND METHODS

### Subjects

All protocols involving human subjects were approved by the Institutional Review Committee of Kagoshima University Dental School, and the study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983. Seventy-five subjects (Table 1) living at home, aged 2 to 18 years, were recruited from a population of Down's syndrome children seen on an ongoing basis in the Kagoshima Prefectural Child Clinic and Guidance Center. Forty-four subjects had only a primary dentition. The definitive diagnosis of Down's syndrome (21 Trisomy) in these subjects was made by chromosomal examination.

### Gingival Health

Gingival inflammation was assessed by a Modified Total PMA Index (M-PMA) of all sites based on the PMA Index of Massler.<sup>12</sup> This index evaluates the number of gingival units (papillary, marginal, and attached gingiva) demonstrating gingival inflammation around erupted teeth and is limited to the anterior gingiva. Originally designed to survey large groups for epidemiologic studies, it is a useful index in the examination of children in whom later stages of periodontal disease are not usually observed. Plaque scores were evaluated by a single dentist using the Oral Hygiene Index by Greene and Vermillion.<sup>13</sup>

### Serum Specimens

Five ml of blood was collected from the antecubital fossa into vacuum tubes without anticoagulants. After clotting, the sera were removed and stored at -70°C until analyzed.

### Antigen Preparation

*Porphyromonas gingivalis* (Pg, ATCC 33277), *Prevotella intermedia* (Pi, ATCC 33563), *Treponema denticola* (Td, ATCC 35405), *Fusobacterium nucleatum* (Fn, ATCC 49256), *Selenomonas sputigena* (Sel, ATCC 43527), *Actinobacillus actinomycetemcomitans* (Aa, ATCC 43718 [Y4]) and *Streptococcus mitis* (Mi, ATCC 9811) were used as sources of antigen in this study. Five-hundred ml batches of these microorganisms were grown in Schaedler broth supplemented with 5µg/ml of hemin and 1µg/ml of menadione in an anaerobic chamber (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) at 37°C. The microorganisms were harvested by centrifugation (13,000 × g) at late log phase in the presence of protease inhibitors (2mM EDTA, 0.5 mM PMSF; 10<sup>-7</sup>M pepstatin A, 0.5mg/ml leupeptin). The cell pellets were washed three times with phosphate buffered saline (PBS; 0.05 sodium phosphate, 0.15M NaCl, pH 7.4) containing 1mM EDTA and then fixed by incubation with 0.5% buffered formalin at room temperature for 16 to 18 hours on a rotary shaker. The formalinized bacteria were washed three times with PBS, then resuspended in distilled water and lyophilized.

### Antibody Measurements

Semiquantitative measurements of the anti-bacterial antibodies in the sera of these subjects against the panel of seven microorganisms were performed using the micro-ELISA; levels of antibody were expressed as ELISA units.<sup>14,15</sup> Briefly, formalinized microorganisms were diluted in 0.1M sodium carbonate buffer to a final concentration of 1 to 10 µg/ml, dispensed into 96-well microtiter plates (0.1ml/well), and incubated for 2 hours at 37°C. The plates were then stored at 4°C until used. Before use, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T).

Serial dilutions of patient sera in PBS-T (beginning with 1:100 for IgG and 1:20 for IgM) were added to the wells and incubated for 1 hour at 37°C. After washing with PBS-T, 0.1ml (1:1,000 dilution) of γ-chain or µ-chain-specific goat anti-human immunoglobulin conjugated to alkaline phosphatase<sup>‡</sup> was added to each well and incubated for 1 hour at 37°C. After a final wash, alkaline phosphatase substrate was added to each well, and the absorbance at 405nm was measured using a multichannel spectrometer. A reference serum composed of a pool of 20 healthy adult sera possessing antibodies to all of the antigens used in this study was also titrated in each

<sup>‡</sup>Zymed Laboratories, Inc., S. San Francisco, CA.

**Table 2. The Prevalence of Gingival Inflammation in Down's Syndrome and Normal Children by Chronological Age Category**

Age Category (Years)	Mean Age	Down's (DS)		Mean Age	Normal (NC)		Significance
		(n)	%		(n)	%	
<4	2.9	(8)	100.0	2.9	(24)	79.2	DS > NC, $P < 0.05^*$
≥4 and <7	5.0	(32)	93.8	5.6	(50)	96.0	N.S.
≥7 and <10	7.9	(21)	86.4	8.4	(55)	100.0	N.S.
>10	11.8	(14)	100.0	14.0	(171)	98.2	N.S.

\*Significant difference.

plate to allow internal standardization and calculation of ELISA units (EU).<sup>14</sup> This reference represented the normal adult antibody levels to the selected microorganisms. EUs were calculated by relating absorbance values from each experimental sample to the reference serum which was assigned a value of 100 EU.

### Statistical Analysis

The relationship between the antibody titer to each microorganism and dental age was evaluated by Pearson's correlation coefficient and Fisher's protected least significant difference (PLSD). The differences in antibody titer stratified by extent of gingivitis were evaluated by Spearman's rank correlation. The subjects were divided into three groups based on their Hellman Dental Age: G1 (HDA IIA, deciduous dentition), G2 (HDA IIC), and G3 (HDA IIIA-IVA, mainly mixed dentition).<sup>16</sup> The dental age better reflects biological development than does chronological age, especially in Down's syndrome in which tooth eruption is delayed compared to normal siblings.<sup>17</sup> Also, the dental age allows a better definition of the relationship between tooth eruption (i.e., deciduous and permanent teeth) and microbial/immunological measurements. The associations of M-PMA and antibody titer with age were examined, and the relationships between M-PMA and plaque score and antibody titer by all subjects and by HDA categories were also determined by regression analysis. In addition, the differences in M-PMA stratified by HDA categories were examined. All statistical analyses were performed on a computer using a statistical software program.<sup>8</sup>

## RESULTS

### Clinical Measurements

The prevalence of gingival inflammation in Down's syndrome in various dental age groups was as follows: <4 years, 100%; between 4 and 7 years, 93.8%; from 7 years to <10 years, 86.4%; >10 years, 100%. A comparison of the prevalence of gingival inflammation in 75 DS versus 300 healthy non-DS children (177 males and 123 females) living in the same region is shown in Table 2 (data

**Table 3. Mean Antibody Titers by Hellman Dental Age of Down's Syndrome Children**

Antigen	HDA	IgG	IgM
		Mean ± SD	Mean ± SD
<i>Pg</i>	G1	77.6 ± 43.4	49.3 ± 31.5
	G2	105.3 ± 147.5	36.8 ± 11.2
	G3	173.9 ± 219.5	43.7 ± 29.7
<i>Aa</i>	G1	234.3 ± 281.9	49.1 ± 29.5
	G2	202.2 ± 107.6	68.8 ± 23.6
	G3	346.0 ± 392.3	47.4 ± 18.7
<i>Fn</i>	G1	114.0 ± 92.6	45.2 ± 29.4
	G2	118.2 ± 70.3	58.7 ± 30.2
	G3	117.8 ± 79.6	43.0 ± 32.6
<i>Pi</i>	G1	100.5 ± 55.4	42.5 ± 38.6
	G2	95.5 ± 73.0	53.0 ± 31.5
	G3	118.5 ± 65.8	38.3 ± 20.0
<i>Sel</i>	G1	71.7 ± 38.9	23.7 ± 15.6
	G2	70.2 ± 75.2	28.1 ± 16.1
	G3	82.1 ± 64.4	28.0 ± 13.6
<i>Td</i>	G1	70.5 ± 43.2	30.4 ± 19.4
	G2	65.7 ± 73.7	30.3 ± 9.5
	G3	66.5 ± 43.4	38.5 ± 22.9
<i>Mi</i>	G1	179.4 ± 120.5	54.0 ± 30.1
	G2	164.4 ± 110.5	62.1 ± 24.0
	G3	206.8 ± 160.4	51.6 ± 20.4

from Morinushi and Ueda, unpublished). As shown, the prevalence of gingival inflammation in DS under 4 years was significantly ( $P < 0.05$ ) higher than that found in normal children.

In the present study, a significant positive correlation ( $r = 0.548$ ,  $P < 0.0001$ ) existed between the M-PMA score and plaque score for G1 subjects (deciduous dentition). M-PMA increased significantly ( $r = 0.397$ ,  $P < 0.001$ ) with chronological age (all DS children). When compared by dental age categories, the average M-PMA score at G3 was higher than at G1, but was not significant ( $P = 0.097$ ).

### Antibacterial Antibody Titers

Serum samples obtained from the DS children were examined for the presence of IgG and IgM antibodies to the panel of seven oral microorganisms. The relationship between antibody titers and dental age category is shown in Table 3. At G1, the average antibody titer to *Aa*, *Mi*, and *Fn* exceeded that of the normal adult reference serum pool (100 EU). The mean IgG antibody titers to *Pg* at G3 (HDA classification corresponding to early puberty) were

\*StatView, Abacus Concepts, Inc., Berkeley, CA.

**Table 4. Relationship Between Antibody Titer and Gingival Inflammation in Down's Syndrome Children**

Antigen	Gingival Inflammation (M-PMA Score)					
	(n)					
	0 (6)	0< and <30 (27)	30≤ and <60 (21)	60≤ and <90 (15)	≥90 (6)	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
<i>Pg</i>	IgG	62.1 ± 37.9	104.3 ± 154.9	115.3 ± 123.5	84.5 ± 47.2	229.2 ± 266.6*
	IgM	44.1 ± 31.9	54.1 ± 33.4	43.3 ± 30.8	40.7 ± 19.4	37.3 ± 24.2
<i>Aa</i>	IgG	168.6 ± 55.6	250.2 ± 347.0	262.5 ± 229.7	278.9 ± 213.1	403.3 ± 646.3
	IgM	50.1 ± 28.9	51.1 ± 23.0	47.1 ± 32.9	58.0 ± 23.5	44.1 ± 24.6
<i>Fn</i>	IgG	63.5 ± 22.7	117.2 ± 106.2	124.7 ± 61.6	115.5 ± 73.0	128.6 ± 124.0
	IgM	55.3 ± 35.6	50.9 ± 32.7	37.9 ± 26.5	48.0 ± 31.6	37.5 ± 25.0
<i>Pi</i>	IgG	118.0 ± 95.6	84.4 ± 35.6	114.2 ± 74.3	117.8 ± 51.8	126.3 ± 69.0
	IgM	28.3 ± 18.5	39.7 ± 22.5	50.0 ± 44.8	46.3 ± 38.9	31.7 ± 14.7
<i>Sel</i>	IgG	75.9 ± 37.5	65.0 ± 38.1	82.0 ± 64.1	66.9 ± 25.1	110.0 ± 99.7
	IgM	19.6 ± 16.0	28.6 ± 16.5	22.8 ± 15.3	25.0 ± 10.2	27.7 ± 17.3
<i>Td</i>	IgG	40.0 ± 23.6	68.8 ± 35	82.9 ± 58.5	57.2 ± 26.6	76.7 ± 52.0
	IgM	31.0 ± 22.8	36.7 ± 21.5	26.9 ± 16.3	29.2 ± 8.0	47.3 ± 33.7
<i>Mi</i>	IgG	149.3 ± 136.5	165.0 ± 90.7	181.5 ± 95.8	223.3 ± 161.7	242.0 ± 273.6
	IgM	49.6 ± 30.9	55.0 ± 22.3	55.7 ± 30.5	56.1 ± 30.5	44.5 ± 22.5

Mean age ± SD of each group: score = 0, 6 ± 1.67; 0< score <30, 6 ± 2.50; 30≤ score <60, 7.1 ± 2.93; 60≤ score <90, 7.3 ± 3.28; score ≥90, 10 ± 5.02.

\*Significant difference compared to titers of individuals having an M-PMA score of "0" (first column).

significantly higher when compared to G1 (corresponding to preschool children). In addition, the mean IgG antibody titer to *Aa* at G3 was significantly ( $P < 0.05$ ) higher when compared to G1 and G2.

When the relationship between the IgG antibody titer and chronological age in DS children was examined, we found that antibody titers to *Pg* ( $r = 0.429$ ,  $P < 0.01$ ), *Aa* ( $r = 0.360$ ,  $P < 0.01$ ), *Fn* ( $r = 0.0278$ ,  $P < 0.02$ ), *Sel* ( $r = 0.279$ ,  $P < 0.02$ ), and *Mi* ( $r = 0.320$ ,  $P < 0.01$ ) increased significantly with age. When IgM titers were examined, an increase was observed only with *Td* ( $r = 0.295$ ,  $P < 0.01$ ).

#### Relationships Between Antibody Titer and M-PMA

When the correlation between antibody titer and M-PMA score was examined in all DS subjects, IgG antibodies to *Pg* ( $r = 0.283$ ,  $P < 0.02$ ), *Aa* ( $r = 0.282$ ,  $P < 0.02$ ), *Sel* ( $r = 0.260$ ,  $P < 0.05$ ), and *Mi* ( $r = 0.330$ ,  $P < 0.05$ ) increased significantly with increasing M-PMA. When the DS subjects were stratified by dental age, we found a significant positive correlation between M-PMA and IgG antibody titers to *Aa* ( $r = 0.496$ ,  $P < 0.02$ ), *Fn* ( $r = 0.569$ ,  $P < 0.01$ ), *Sel* ( $r = 0.567$ ,  $P < 0.01$ ), and *Mi* ( $r = 0.666$ ,  $P < 0.01$ ) in G3. In addition, plaque scores were highest in G3.

When the mean antibody titers, grouped by extent of gingival inflammation (M-PMA), were compared (Table 4), we found no significant differences in either the IgG or IgM antibody titers to any of the microorganisms (Spearman's rank correlation). However, the mean IgG antibody titer to *Pg* was significantly higher (Fisher's PLSD) in the most extensive disease category compared to the "0" category. The mean IgG antibody titers to *Aa* and *Mi* were also higher in the most extensive disease

category compared to the other categories, but this difference was not found to be statistically significant (Fisher's PLSD).

#### DISCUSSION

Previous studies of normal subjects have demonstrated that the serum antibody responses to a number of oral microorganisms correlated to the severity of periodontal disease.<sup>18-30</sup> Other studies have reported that serum antibody levels to periodontopathic microorganisms are elevated in subjects having gingivitis.<sup>23,27,28</sup> However, there is a paucity of data relating the antimicrobial serology of gingivitis to antibody response in children with Down's syndrome (DS), a population presenting with a high prevalence of rapidly progressing extensive gingivitis starting in childhood.<sup>1-3</sup> In this light, the findings of our study are important since they begin to clarify the relationship between the microbiota and gingivitis associated with Down's syndrome.

We found that the prevalence and extent of gingival inflammation (as measured by M-PMA) in DS is significantly higher than in normal children under the age of 4 years. The higher prevalence and greater extent of gingival inflammation in DS under the age of 6 years, compared with normal children, confirms previous studies.<sup>31</sup> The relationship is especially marked in G1, representing children below the age of 6 years. One possible explanation for this observation is that the early onset of gingival inflammation is due to the reported abnormalities of systemic host defense,<sup>5,6</sup> perhaps resulting in earlier colonization by periodontopathic microorganisms.

Previous studies of DS in children aged 4 to 18 years did not evaluate periodontopathic microorganisms in subgingival plaque, nor did they examine serum antibodies

to periodontopathic microorganisms. Of the few studies examining the incidence of periodontopathic microorganisms in DS children, Meskin et al.<sup>8</sup> studied children ranging from ages 5 to 12 years presenting with extremes in periodontal pathology. They concluded that there was an association between the presence of black-pigmented *Bacteroides* (BPB) and the prevalence of periodontal disease in DS children. In another study, Cutress et al.<sup>10</sup> were not able to detect significant differences in BPB counts when DS and subjects with mental retardation (aged 9 to 24 years) were compared. However, there was a significant difference in BPB counts between subjects living at home and those who were institutionalized. Barr-Agholme et al.<sup>11</sup> reported that *A. actinomycetemcomitans* was present in the subgingival plaque of DS subjects at a significantly greater frequency than in healthy children. They explained the higher prevalence of *A. actinomycetemcomitans* among DS subjects based on similarities with *Haemophilus influenzae*, which is associated with respiratory infections in DS.<sup>32</sup> DS patients are infected early with *H. influenzae*, and since *A. actinomycetemcomitans* is related to some species of the *Haemophilus* genus, they hypothesized a generalized susceptibility to these species. However, this is an insufficient explanation for the high prevalence of rapid progression and extensive gingival inflammation starting in early childhood of DS. The temporal relationships involved in colonization by microorganisms associated with periodontal disease and the relationship between gingival inflammation and periodontopathic microorganisms require additional investigation.

Our observations of the changes in IgG and IgM antibody titers with age suggest that colonization levels of *Pg*, *Aa*, *Fn*, *Sel*, *Td*, and *Mi* increase with age. We are unaware of studies examining changes in antibody titers in healthy children. However, previous studies of colonization indicate that the incidence of black-pigmented *Bacteroides* (*Bacteroides melaninogenicus* or *Bacteroides intermedius*) in the supragingival plaque of young children increases 15% to 40% over a 3- to 6-year period.<sup>32-35</sup> In a study by Frisken et al.<sup>36</sup> BPB species were detected in the subgingival plaque of 31% to 61% of children aged 5 to 7 years, but *Pg* was detected in only 1.6% of the children. *Fn* was detected in 62% of children and *Aa* detected in 3% to 33% of children the same age range (varies with method of isolation). *Pg* and *Aa* were not detected routinely in children under 3 years. Conversely, *Fn* was detectable within the first 1 to 6 months of life.<sup>37</sup>

Using antibody titer as a surrogate for microbial colonization, some information on the colonization by the seven microorganisms may be deduced. At G1 (preschool children), titers to *Aa*, *Mi*, and *Fn* were significantly higher than the adult pool, while the titer to *Pi* was almost the same. This suggests that *Fn*, *Aa*, *Mi*, and *Pi* are probably present in significant numbers in G1 children. These trends are in agreement with the age-related colonization

trends reported for non-DS children of children reviewed above.

The extent of gingivitis, as evaluated with the M-PMA, increased with age in this study. We observed that antibody titers to *Pg* (IgG) and *Aa* (IgM) were significantly elevated in the most diseased subjects. Similarly, at G3 (early puberty) these titers were significantly higher compared with G1 (preschool children) or G2. In addition, IgG antibody titers to *Pg*, *Aa*, *Fn*, *Sel*, and *Mi* correlated significantly with M-PMA in the G1 age group.

Some caution regarding the use of antibody titers as a surrogate for microbiological colonization is warranted. Clearly, there is not necessarily a direct relation between titer and bacterial presence. A progressive age-related correlation with antibody titer might also reflect immunological maturation in the young child. However, at least in the G1 Down's children where the titers are significantly elevated, the differences are unlikely to be related to immunological maturity.

Our findings suggest that colonization by *Aa* and *Fn* is associated with the onset and extent of gingivitis in DS children under the age of 5 years. In contrast, studies of non-DS children indicate that *Pg* is closely associated with the extent of gingivitis at puberty, probably associated with changes in levels of sexual hormones such as estradiol.<sup>38-41</sup> *Sel* appears to be associated with deteriorating periodontal health (simple gingivitis to severe periodontitis), based on our findings and reports by Moore et al.<sup>42,43</sup> Finally, colonization by *Mi* in DS children appears to correlate with the extent of gingivitis. This may be important based on the work of Nagata et al.,<sup>44</sup> which indicated that *Mi* may play a role in the colonization of the periodontal pocket by *Pg*. We are aware that these associations need to be further evaluated and the actual presence of the microorganisms confirmed. However, this work represents the first attempt at establishing the differences between DS and non-DS children with regard to colonization by putative periodontal pathogens.

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