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Serotype and Leukotoxic Strain Distribution of *Actinobacillus (Haemophilus) Actinomycetemcomitans* in Korean Localized Juvenile Periodontitis

Hyun-Ju Chung[1], Chong-Pyoung Chung[2] and Seong-Heui Son[2]

[1]*Department of Periodontology, College of Dentistry, Chonnam National University*
[2]*Department of Periodontology, College of Dentistry, Seoul National University*

=국문초록=

한국인 국소 유년성 치주염환자의 *Actinobacillus(Haemophilus) Actinomycetemcomitans* 혈청형 및 백혈구독성 균주 분포

전남대학교 치과대학 치주과[1] 및 서울대학교 치과대학 치주과[2]

정현주[1]·정종평[2]·손성희[2]

국소적 유년성 치주염의 원인균으로 중요시되고 있는 *Actinobacillus actinomycetemcomitans(Aa)*는 구미인의 병소에서 혈청형 b형이 주종을 이루는 것으로 보고되었으나, 한국인에서의 부분적인 분리균주는 c형이 빈번한 것으로 관찰되었다.

이에 본 연구는 16명의 국소적 유년성 치주염 환자에서 *Aa*의 발현빈도를 조사하고 혈청형별로 분류하여 그 분포 및 백혈구 독성을 평가하기 위하여 시행되었다. 치주낭 및 건강치은 열구에서 보존치료용 paper point를 이용하여 치은연하 치태세균을 채취하여 *Aa*의 선택배지에 도말한 후 10% 탄산가스 배양기에서 3~5일간 배양하였으며, 집락의 형태, catalase검사, Gram염색, 생화학검사로써 분리·동정하였다. 가토에서 3가지 혈청형의 표준균주인 ATCC 29523(a) Y4(b) SUNYaB67(c)에 대한 항혈청을 얻은 후 황산암모늄 침전법과 면역흡착법에 의하여 특이성을 갖는 감마글로불린액을 얻어서 ELISA법에 의해 분리균주의 혈청형을 분류하였다. 백혈구 독성은 다형핵 백혈구와 *Aa*분리균주를 함께 배양한 후 상층액에 대한 lactate dehydrogenase의 활성을 측정함으로써 평가하였다.

그 결과는 다음과 같다.

1. *Aa*균은 국소적 유년성 치주염 환자 16명중 75%에서 발견되었으며, 병소부위의 71%, 그리고 정상부위의 6%에서 나타났다.
2. 국소적 유년성 치주염 병소의 임상적 양상은 병소내 *Aa*의 존재여부에 따른 차이를 보이지 않았다.
3. 3가지 혈청형의 환자별 분포는 9명의 환자에서 유사하게 관찰되었으며, 3명의 환자에서는 동일한 구강내 또는 동일한 치주낭에서 다른 2가지 혈청형이 함께 분리되었다.
4. 백혈구 독성검사를 시행한 46개 균주중 22%에서 독성을 나타냈으며, 채취부위의 69%에서 백혈구 독성균이 존재하였다. 또한, 동일한 병소에서 독성균과 비독성균이 함께 관찰되었다.
5. 백혈구 독성균의 분포는 3가지 혈청형간에 차이를 인정할 수 없었다.

Key Words: Juvenile periodontitis; *A. actinomycetemcomitans* serotype; leukotoxicity.

INTRODUCTION

Localized juvenile periodontitis (LJP) is a form of periodontitis that results in comparatively rapid alveolar bone loss localized to permanent incisors and first molars in young individuals, predominantly teenagers.[4,41] This form of periodontitis was formerly described as periodontosis because it was thought to be degenerative entity. Recently,

several investigators have demonstrated an association between LJP and bacterial species, especially capnophilic, Gram-negative rods.[33,39,45,55] One species was later classified as new genus, *Capnocytophaga*[22] and the other was identified as *Actinobacillus actinomycetemcomitans* by Tanner *et al.*[57] who reported that *A. actinomycetemcomitans* was associated with rapidly progressing alveolar bone loss in young individuals.

The importance of *A. actinomycetemcomitans* as an etiological agent in LJP has been supported by the evidences: 1) association studies demonstrated that almost all LJP lesions harbored high numbers of *A. actinomycetemcomitans* while this organism was found less often and in much lower numbers in other sites;[1,27,40,51,53] 2) there were many reports correlating successful treatment of LJP with elimination of *A. actinomycetemcomitans* from periodontal pockets and recurrence of the disease related to the appearance of the organism;[7,9,20,28,51,52,63] 3) histopathologic findings revealed *A. actinomycetemcomitans* invading the gingival connective tissue in LJP lesions;[8,17,38] 4) immunological studies demonstrated elevated humoral immune response to this organism in LJP patients[14,15,24,35,58] and; 5) it was described that a number of *A. actinomycetemcomitans* pathogenic products were capable of both inhibiting protective immune mechanism and causing periodontal tissue destruction.[2,18,34,37,43,56,58,60,65]

Of particular interest regarding the pathogenic potential of *A. actinomycetemcomitans* is the presence of leukotoxic factor in certain strains.[2,56,58,65] Although the role of the leukotoxic factor in the pathogenesis of juvenile periodontitis was not known, this factor could affect the normal function of polymorphonuclear leukocyte (PMNL) which is key cell in defense against infectious agents. It was reported that not all *A. actinomycetemcomitans* produced significant level of leukotoxic factor and some strains were variable producers.[2] Both leukotoxic and non-leukotoxic *A. actinomycetemcomitans* were isolated in the same site, and in proportions related to periodontal status.[53,65]

On the other hand, serological investigations of *A. actinomycetemcomitans* have identified specific serotypes and bacterial antigens which may be important in the etiology of LJP. King and Tatum[19] reported 3 serogroups of non-oral *A. actinomycetemcomitans* on the basis of a heat-labile component and Zambon *et al.*[66] distinguished 3 serotypes (a,b,c) and reported that periodontally healthy subjects harbored serotype a and b in approximately equal prevalence, and the serotype b was increased in LJP, implicating the serotype b antigen as particularly important in the pathogenesis of this form of periodontal disease.[53, 65]

Preliminary studies from our laboratory have shown that among the twelve selected *A. actinomycetemcomitans* strains from 7 LJP paients, nine strains belonged to serotype c[55] and humoral immune response was higher to SNUDC 10-1 (c) than to Y4 (b).[10,23] Furthermore, normal juveniles had higher antibody titers against SNUDC 10-1 than Y4, too. These results suggested that LJP patients in Korean might harbor *A. actinomycetemcomitans* of different serotype from Caucasian LJP patients due to different diet habit and genetic environment.

The purpose of present study was to investigate the prevalence and serotype distribution of *A. actinomycetemcomitans* in localized juvenile periodontitis and to evaluate the leukotoxicity of clinical isolates as an important pathogenic factor and relate it to the serotype of isolates.

MATERIAL AND METHOD

A. Subject Selection

From the dental clinic of Seoul National University Hospital and affiliated hospitals, a total of 16 patients were selected. The subjects were between the ages of 18 and 33 years and diagnosed as LJP according to Baer's criteria.[4] They were chosen for the study based on the absence of evidence of systemic disease from their medical history and systemic antibiotic therapy or periodontal manipulation including oral prophylaxis in the previous 6 months.

B. Clinical measurements

From each patient both disease site and control (relatively healthy) site were included. The disease site had at least 4mm loss of attachment in incisors or molars and the control site was selected on the basis of the absence of clinical or radiographic evidence of bone loss. Clinical parameters at each site included Plaque Index (Silness & Löe),[44] Sulcus Bleeding Index (Mühlemann & Son),[32] gingival crevicular fluid amount,* probing depth and loss of attachment from cementoenamel junction. Probing depth was measured to the nearest half millimeter with a standard probe.

C. Microbiological sampling and identification

The examined tooth was isolated with cotton rolls and supragingival plaque was removed with sterile cotton pledgets. Three fine sterile paper points* were then inserted into the periodontal pocket until resistance was met and left in place for 10 sec. Paper points from each site were transferred into the tube containing 2ml of sterile Ringer's solution. The bacteria were dispersed using a Vortex mixer for 60 sec and serially diluted in 10-fold steps in 9ml of the Ringer's solution. Samples of 100μl of suitable dilution were plated onto the selective medium for *A. actinomycetemcomitans* and *Capnocytophaga*, and nonselective medium for black-pigmented *Bacteroides* and for total anaerobic colony count. The selective medium for *A. actinomycetemcomitans* consisted of tryptic soy agar,# 0.1% yeast extract,# 10% horse serum,@ 75 μg bacitracin and 5 μg vancomycin¢ per ml.[47]

The selective medium for *Capnocytophaga* consisted of tryptic soy agar,# 5% rabbit blood, 0.1% yeast extract,# 50 μg vancomycin and 100 μg polymyxin¢ per ml.[29]

Tryptic soy agar supplemented with 5% rabbit blood, 5 μg hemin per ml and 0.5μg menadione per ml was used as a nonselective medium. The seeded selective and nonselective media were incubated in 10% CO_2 chamber and anaerobic chamber, respectively.

After 3 to 5 days incubation, *A. actinomycetemcomitans* was identified on the basis of colony morphology and catalase activity. *Capnocytophaga* was also identified on the basis of colony morphology and bacterial motility in phase contrast microscopy. The appropriate dilution was chosen for each medium such that 30 to 100 colonies were evenly distributed on the plate to be counted. All colonies on the chosen plate with the most suitable dilution were counted using the total area of each plate. The proportion of three bacteria was expressed as percentage in the total colony count on the nonselective plate. Suspected *A. actinomycetemcomitans* isolates were subcultured and confirmed by selected biochemical tests[33,51] and grown into thioglycolate broth and stored at -70°C until used.

D. Serotyping of *A. actinomycetemcomitans* isolates

1) Antisera production

Whole cells of *A. actinomycetemcomitans* reference strains+ of 3 serotypes were used as immunogens. These strains were grown in the fluid thioglycolate broth at 37°C in CO_2 incubator for 48h. The cells were harvested by centrifugation (16,000xg, 15min, 4°C) and washed three times in sterile PBS, pH 7.2, and suspended in sterile saline to a concentration of 10mg wet weight per ml. New Zealand white rabbits weighing 2.5-3.0Kg were injected intravenously *via* the marginal ear vein with increasing doses of bacterial suspension from 0.5 to 2.0 ml, every other day for a total of 8 injections. One week rest was followed by a booster series of 3 consecutive daily injections giving 2.0ml of bacterial suspensions. Trial blood samples were obtained from the central ear artery and the antibody titers were determined by indirect immunofluorescence.

*Harco Periotron 6000, Harco Electronics Ltd., Canada

* Johnson & Johnson, East Windor N. J., U.S.A.

Difco Lad., Detroit Mich., U. S. A.

@ Gibco, Grand Island Biological Co., Grand Island, U. S. A.

¢ Sigma Chemical Co., St. Louis, Mo, U. S. A.

+ ATCC 29523 (serotype a)
Y4 (serotype b)
SUNY aB 67 (serotype c)

After a satisfactory antibody titer had been obtained, the rabbits were terminally bled by cardiac puncuture. The antisera were separated and heated to 56°C for 30 min and stored at -20°C.

2) Ammonium sulfate precipitation of gamma globulins

For the fractionation of gamma globulins from antisera, repeated precipitation with saturated ammonium sulfate solution was performed at a final concentration of 50% saturation.

3) Immunoabsorption

The ammonium sulfate-fractionated antisera were absorbed with *A. actinomycetemcomitans* whole cells of the heterogenous serotypes until they reacted specifically only to the immunogens. Whole cells (100mg wet weight) were added to 1ml of rabbit antisera and placed in a shaker at 37°C for 1 h and kept it at 4°C for 12h. The mixture was centrifuged (16,000xg, 60min) and the antisera supernatant was removed. The absorption was repeated, and the absorbed antisera were aliquoted and stored at −70°C until used. The specificity was confirmed by indirect immunofluorescence. The quantitative determination of gamma globulins in the immunoabsorbed antisera was done with U-V spectrophotometer.*

4) Serotyping by ELISA

Washed whole cells of *A. actinomycetemcomitans* isolates were suspended in 0.1M $NaCO_3$ buffer at pH 9.6 containing 0.02% NaN_3 (antigen buffer) and the concentration adjusted to an absorbance of 0.25 to 0.3 at 580nm. Antigen suspension was added to each well of polystyrene microtiter plates@ and incubated at 37°C for 2h, and the antigen plates were stored at 4°C until used. The assay procedure included 3 steps; 1) incubation of washed whole cell-coated plates with serotype specific antisera diluted in PBS containing 0.05% Tween 20 and 0.02% NaN_3 (antibody buffer) for 2h at 37°C, 2) washing (three times with 0.9% NaCl, pH7.4 containing 0.05% Tween¢ 20: washing buffer) followed by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase# for 16-18h at room temperature, and 3) addition of ρ-nitrophenyl phosphate¢ (1mg/10ml in 0.05M $NaCO_3$, pH 9.8 with 1mM $MgCl_2$: substrate buffer) as a substrate. The reactions were terminated at 30 min by additon of 1N NaOH and the extent of reaction was determined spectrophotometrically at 400nm in a micro ELISA reader.@

E. Assay for leukotoxicity

Test strains included ATCC 29523, Y4 and SUNY aB 67 as reference strains and our oral isolates. All strains were grown in brain heart infusion broth to early stationary growth phase, harvested by centrifugation, washed in PBS, pH 7.2, suspended in Hank's balanced salt solution (HBSS) and adjusted to an absorbance ranging from 0.2 to 1.0 (2×10^8 to 1×10^9) at 650 nm spectrophotometrically.

Human peripheral blood PMNL were isolated from heparinized venous blood of healthy donors by dextran sedimentation, followed by Ficoll-Hypaque density gradient centrifugation. The isolated PMNL were suspended in HBSS to a density of 2×10^7 cells per ml.

Bacterial cells were incubated in a water bath under constant shaking with PMNL in HBSS resulting in a total volume of 1ml at 37°C in duplicates. Controls consisted of PMNL in HBSS alone. At the end of the experiment, cultures were placed on ice for 10 min to suppress the leukotoxic effect and then centrifuged to remove PMNL and bacteria. The release of lactate dehydrogenase (LDH) was used to monitor cytotoxicity. LDH was assayed by a modification of the procedure of Berger and Broida, which depends on the formation of a colored phenylhydrazone as a measure of the amount of substrate (pyruvate) remaining in the standard assay procedure.[5] Samples (100μl) of serially diluted supernatants from cytotoxicity incubation mixtures were added to 0.2ml-aliquots of buffer-substrate prepared according to Sigma kit 500* and incubated at 37°C for 15 min.

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* Gilford Model 2,600, Gilford Instrument Lab. Inc., Oberlin, Ohio, U. S. A.

@ Dynatech Lab. Inc., Alexandria, VA, U. S. A.

Cooper Biochemical Inc., Malvern, PA, U. S. A.

¢ Sigma Chemical Co., St. Louis, MO, U. S. A.

*Sigma Chemical Co., St. Louis, MO, U. S. A.

Table 1-a. Clinical parameters and bacterial distribution in LJP lesion

Patient	Age/Sex	Site	SBI	GCF	PI	PD	B.P.B.*	*Aa**	*Capnocytophaga**
A.Y.H.	18/F	1	3	99		5		4.88	47.78
L.M.S.	20/F	6	1	200	2	8	0	8.00	0
		7	2	189	1	7	0	10.00	0
		6	1	46	0	8		+	0
		1	2	110	2	5		+	0
Y.J.U.	20/F	6	2	186	1	9	24.53	0.56	0.56
		1	1	137	0	5	0	0	0
		6	1	188	2	10	43.60	0	0
		1	2	127	1	8	7.81	1.56	0
M.H.T.	20/M	6	1	94	1	5	0.88	2.25	0.13
		6	1	125	0	6	0.03	13.50	4.00
		6	1	106	0	7	4.33	0.83	0
W.G.Y.	21/F	6	1	200	1	9		0	
		1	3	87	1	7		0	
		6	2	200	1	7		+	
C.Y.S.	28/F	6	2	134	0	5		55.63	0
S.J.K.	30/M	1	1	99	0	7		0	0.40
P.C.J.	26/M	1	3	109	2	6		0.05	0.04
S.K.Y.	28/F	6	3	133	2	6		0	2.00
N.M.K.	24/F	1	2	30	2	4		0	0.03
		6	2	80	2	8		0	0.05
S.Y.S.	33/M	1	1	62	1	6		4.08	0
P.W.K.	33/M	6	2	74	2	4		0	1.50
		1	2	127	2	5		0.20	0
H.M.H.	25/F	6	3	122	0	8		3.50	0
		1	3	128	2	5		0.63	0
S.A.P.	26/F	1	2	43	0	9	0	55.00	0
		6	1	113	2	6	0	25.00	10.71
		6	1	184	3	9	8.38	0	0
W.G.S.	25/F	1	0	69	1	6	0	0	0
		6	1	17	1	4	67.10	0.26	0.44
		6	1	187	2	7	18.20	0	0
L.L.W.	25/F	6	3	161	2	5		0	
		6	2	145	1	6		0	
		6	2	144	1	5		0	

Then 200 μl of the Sigma color reagent (0.02% 2,4-dinitrophenylhydrazine in 1N HCl) was added, and the mixture was held at room temperature for 20 min. A 2.0ml volume of 0.4M NaOH was added with rapid mixing and the absorbance was read against a water blank at 450nm spectrophotometrically.

Total LDH activity in the culture was measured by disruption of PMNL treated with 0.1% Triton X-100. Back ground levels of LDH released spontaneously from PMNL during incubation in HBSS alone for 60 min were substracted from the test results. The results were expressed as the percentage of total

Table 1-b. Clinical parameters and bacterial distribution in control site

Patient	Site	SBI	GCF	PI	PD	B.P.B.*	*Aa**	*Capnocytophaga**
A.Y.H.	4	1	38		3		0	0
L.M.S.	3	0	11	1	2	0	0	0
Y.J.U.	1	0	39	1	2	2.17	0	0
M.H.T.	3	1	13	0	3	1.39	0	5.83
W.G.Y.	3	1	12	0	3		0	
C.Y.S.	4	0	37	0	3		0	0
S.J.K.	3	0	43	0	2.5		0	0
P.C.J.	3	1	38	1	2.5		0.17	0.67
S.K.Y.	1	0	50	1	3		0	0
N.M.K.	3	2	10	1	3		0	0
S.Y.S.	4	0	23	0	2		0	0
P.W.K.	3	0	8	1	2.5		0	0
H.M.H.	3	1	16	1	2.5		0	
S.A.P.	3	1	42	0	2.5	0	0	0
W.G.S.	4	0	18	0	3	0	0	0
L.L.W.	3	0	16	0	3		0	

Abbreviations used in Table 1-a, 1-b:
LJP; localized juvenile periodontitis
SBI, Sulcus Bleeding Index (Mühlemann & Son[32])
GCF; gingival crevicular fluid
PI; Plaque Index (Silness & Löe[44])
PD; probing depth
BPB; black-pigmented *Bacteroides*
Aa: Actinobacillus actinomycetemcomitans
"+" means detected but the proportion was not determined.
*Values are in percentages in the total colony count

Table 2. Prevalence of *A. actinomycetemcomitans*

	Prevalence in subject (%)	Prevalence in site (%)	Proportion of *Aa* in culture positive sites
Control	1/16(6)	1/16(6)	0.17±0.01
L J P	12/16 (75)	20/35(71)	10.94±0.95*

* The values in mean ± standard error (percentage).

Table 3. Clinical observation at control and disease sites

	Control sites (n=16)	Disease sites (n=35)
PI	0.87 (0.99)#	1.35 (0.88)
SBI	0.50 (0.63)	1.74 (0.82)*
GCF	25.94(25.40)	122.60 (50.90)*
PD	2.70 (0.40)	6.49 (1.63)*
LA	ND	7.03 (1.90)

* There were statistically significant difference between control and disease sites ($P < 0.01$).
ND: not detected
The values are in mean (standard deviation).

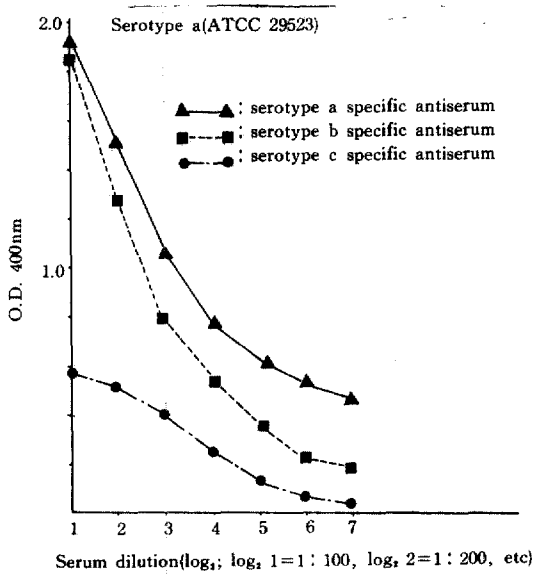

Fig. 1. Response of 3 serotype-specific antisera to the standard strain of serotype a - *A. actinomycetemcomitans.*

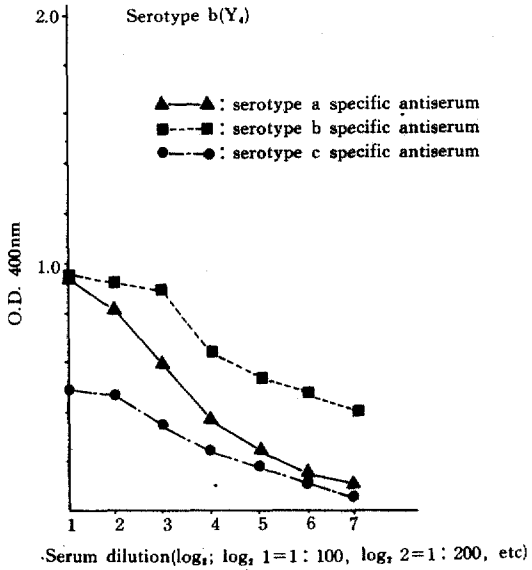

Fig. 2. Response of 3 serotype-specific antisera to the standard strain of serotype b - *A. actinomycetemcomitans.*

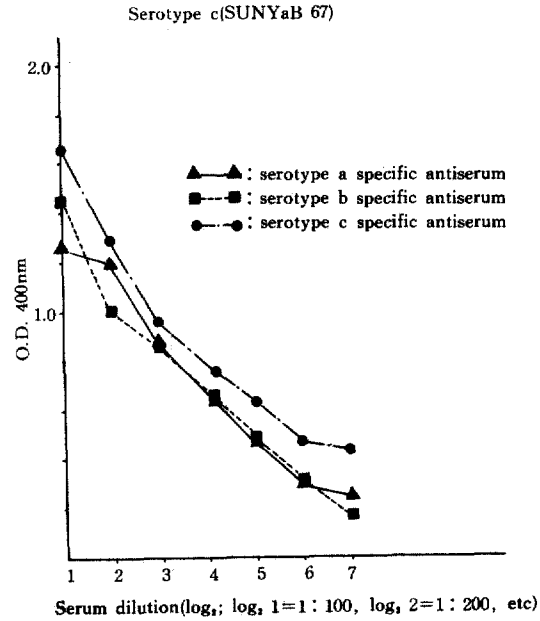

Fig. 3. Response of 3 serotype-specific antisera to the standard strain of serotype c - *A. actinomycetemcomitans.*

black-pigmented *Bacteroides* or *Capnocytophaga* were detected in 8 out of 10 examined sites and T-test for independent observations and the significance level used was 0.05.

RESULTS

A. Clinical measurement and the prevalence of *A. actinomycetemcomitans* in LJP

Clinical parameters and distribution of 3 periodontopathogenic bacteria in each sample site were summarized and no relationship appeared between clinical measurement and bacterial distribution in disease sites (Table 1).

Of 16 patients, 7 patients harbored this species in all their disease sites and 5 patients had 33 to 67% of their disease sites infected with this organism. Remaining 4 patients had no *A. actinomycetemcomitans.*

Thus, this species was isolated in 12 out of 16 LJP patients, resulting in the prevalence of 75%. In 20 out of 35 disease sites and one control site, *A. actinomycetemcomitans* was detected. This organism constituted more than 5% of the total anaerobic count in 6 out of 17 *A. actinomycetemcomitans* -positive disease sites. The mean proportions of this species in culture positive sites were 0.17% in the control sites and 10.94% in the disease sites(Table 2). Among the disease sites without cultivable *A. actinomycetemcomitans,*

Table 4. Mean clinical measurement at disease site with and without cultivable *A. actinomycetemcomilans*

	With cultivable *Aa* (n=20)	Without cultivable *Aa* (n=15)
PI	1.00 (0.82)*	1.47 (0.83)
SBI	1.80 (0.77)	1.67 (0.90)
GCF	117.40 (50.10)	127.90 (52.00)
PD	6.45 (1.50)	6.53 (1.85)
LA	7.25 (1.42)	6.80 (2.61)

* The values are in mean (standard deviation).

Table 5. Serotype distribution of *A. actinomycetemcomitans* in LJP

Patient	Site	No. of isolates	Serotype	Patient	Site	No. of isolates	Serotype
A.Y.H.*	1	1	a	W.G.Y.	6	15	c
		1	b	C.Y.S.*	6	1	b
L.M.S.	6	2	c			5	c
	6	1	c	S.Y.S.	6	1	b
	7	1	c	S.A.P.	1	1	c
	1	1	c		6	5	c
Y.J.U.	6	2	a	A.B.Y.*	6	3	b
	1	1	a		1	3	b
M.H.T.	6	12	a		1	1	a
	6	1	a				
	6	11	a				

* Three patients harbored 2 different serotypes of *A. actinomycetemcomitans*.

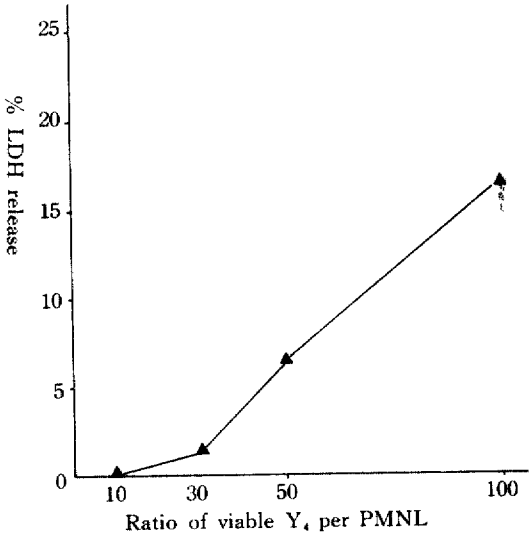

Fig. 4. LDH release from PMNL exposed to various ratios of viable Y4.

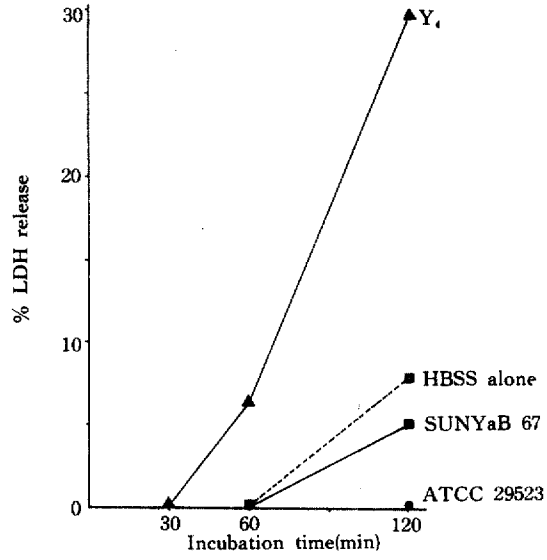

Fig. 5. LDH release from PMNL exposed to viable Y_4, ATCC 29523, and SUNYaB 67 and PMNL in HBSS alone as control for different incubation periods.

Table 6. Prevalence of each serotype of *A. actinomycetemcomitans*

	Serotype a	Serotype b	Serotype c	Total
No. of subjects	4	4	4	9* (12)
No. of sites isolated	7	5	8	18# (20)
No. of isolates	29	9	31	69

* Three subjects habor 2 different serotypes of *A. actinomycetemcomitans.*

\# Two sites have 2 different serotypes of *A. actinomycetemcomitans.*

Table 7. Lactate dehydrogenase release from human PMNL incubated with oral *A. actinomycetemcomitans* isolates

Patient	Site	No. of isolates	LDH(%) release	Patient	Site	No. of isolates	LDH(%)
A.Y.H.	1	1	0	C.Y.S.	6	5	0
		1	10.0			1	10.7
L.M.S.	6	1	6.5	S.Y.S.	6	1	0
M.H.T.	6	3	0	S.A.P.	1	1	0
		1	5.0		6	1	0
	6	1	2.5			1	2.8
	6	4	0			1	10.0
		1	2.5	A.B.Y.	6	3	0
		1	9.0		1	1	0
W.G.Y.	6	15	0			1	4.8
					1	1	0

Table 8 Relationship between serotype and leukotoxicity of *A. actinomycetemcomitans* isolates (Values in parentheses are No. of the disease sites which have strains)

	Serotype a	Serotype b	Serotype c	Total
Leukotoxic	4(3)	2(3)	4(2)	10(8)
Nonleukotoxic	8(2)	6(2)	22(3)	36(7)
Total	12(5)	8(5)	26(5)	46(13)

available LDH activity.

The statistical analysis was carried out with from 2 sites, none of these species was isolated.

As for gingival inflammation, the disease sites showed significantly higher Sulcus Bleeding Index and greater gingival crevicular fluid than control sites did ($P<0.01$).

Periodontal tissue destruction assessed with probing depth and loss of attachment was significantly severe in the disease sites. Plaque accumulation recorded by Plaque Index tended to be higher in the disease sites although the difference was not significant between the control and the disease sites (Table 3).

The mean clinical measurements at 15 disease sites without *A. actinomycetemcomitans* were compared to those at 20 disease sites with this species (Table 4). Although plaque accumulation tended to be higher in the sites without this species, gingival inflammation and probing depth as well as loss of attachment was not different between the disease sites with and without *A. actinomycetemcomitans.*

B. Serotype distribution

Reaction between 3 serotype-specific antisera and 3 serotypes of standard strains was observed by ELISA. The dilution and the absorbance

were plotted on the X-and Y-axis respectively and the straight line of the regression was drawn (Fig. 1. 2 and 3). From these patterns of reaction the working titer of 1:800 to 1:6400 was determined in order to separate the crossreactivity from the specific reaction.

From 1 to 23 isolates were serotyped per individal and a total of 69 isolates from 9 LJP patients were included. Among these patients, 2 patients had 27 isolates of serotype a, 3 patients had 26 isolates of serotype c and 2 patients harbored 2 different serotypes in the same disease site. Of remaining 2 patients, one had an isolate of serotype b and the other harbored different serotypes in different sites (Table 5). Thus the overall patients showed approximately equal proportions of 3 serotypes, and serotype a and c tended to be predominant in the disease sites (Table 6).

There was no relationship between the serotype distribution and clinical observations.

C. Leukotoxicity

Preliminary study using reference strains revealed that LDH activity released from PMNL exposed to viable *A. actinomycetemcomitans* was bacterial dose and incubation time-dependent (Fig. 4 and 5). In this experiment PMNL were incubated with 50 bacteria per PMNL for 60 min. Of 46 isolates from 13 disease sites in 8 patients, 10 strains from 8 disease sites in 6 patients were leukotoxic. A total of 5 disease sites did not show any leukotoxicity of isolates. Thus the prevalence of leukotoxic strain was 62% in sites and 22% of isolated strains. The leukotoxic level assessed with LDH release ranged from 2.5% to 10.7% (Table 7). The presence and extent of leukotoxicity had no relationship with the severity of clinical parameters.

Four strains out of 12 isolates categorized to serotype a, 2 strains out of 8 serotype b-isolates and 4 strains out of 26 serotype c-isolates were leukotoxic. Thus serotype a and b was more frequently leukotoxic than serotype c. However, the site prevalences considering serotype and leukotoxicity were almost similar (Table 8).

DISCUSSION

As for clinicial parameter, plaque accumulation was not significantly different between the disease sites and the control sites. Baer[4] described that local irritant did not correlate with the bone loss and this was observed in our LJP patients. But elder LJP patients tended to show higher plaque accumulation in disease sites than younger patients and in control sites. This finding was consistent with the reports that elder juvenile patients had similar clinical features to adult periodontitis, indicating the possibility of plaque accumulation resulting from difficulty in plaque control in long standing periodontal pocket.[24,42]

Gingival inflammation assessed with Sulcus Bleeding Index and gingival crevicular fluid were significantly higher in the disease sites than in the control sites. Frequent occurrence of bleeding upon probing in consistent with recent study that showed a significant correlation between the severity of attachment loss in LJP and gingival inflammation, and indicated the progressive nature of these lesions.[1] Thus, postulate of Baer did not prevail if bleeding upon probing was included.

Previous studies showed that *Bacteroides, A. actinomycetemcomitans,* and *Capnocytophaga* were observed to be closely related to disease-active periodontitis.[40,48,56] Among them, *A. actinomycetemcomitans* was frequently detected and/mostly in greater proportion in juvenile periodontitis lesion.[40,51,64] In our study, this species was recovered in 75% of LJP patients. This result was similar to previous reports.[1,15,40,51,52] The proportions of *A. actinomycetemcomitans* in the total anaerobic count ranged from 0.05% to 55.63% in LJP lesions. On the other hand, in the control sites the proportion of the bacterium was 0.17% with the prevalence of 6%, which is much lower than that of previous reports.[50,64]

From the longitudinal studies, *A. actinomycetemcomitans* was strongly associated with individual periodontal pocket undergoing active tissue destruction and was found to occur in active sites at levels 100-fold greater than those found in inactive sites.[26,28] Therefore, absence or low pro-

portion of this organism in disease site may reflect a current state of remission, undetectable by clinical observation. When comparing the clinical measurement between the sites with and without cultivable *A. actinomycetemcomitans,* present study revealed no association between clinical status and presence of this organism as shown in Finnish LJP lesions,[1] in contrast to the reports that positive correlation existed between *A. actinomycetemcomitans* and pocket depth and gingival inflammation.[51,62]

The detection of black-pigmented *Bacteroides* and/or *Capnocytophaga* as other periodontopathogen in 80% of disease sites without cultivable *A. actinomycetemcomitans* suggested that the lesions may not be progressive or contain other periodontopathogens which were not examined.

In serological investigation, immunofluorescence[5,25,33] and ELISA[7,12,14,16] have been commonly used. Recently, an ELISA technique for the serological identification of black-pigmented *Bacteroides* species has been described.[13] The investigators showed that *Bacteroides* species could be identified in mixture of microorganisms and further delineated the usefulness of this technique by identifying the organism by species from primary isolation plates of samples from periodontal lesions without biochemical characterization.[11] In our study using an ELISA technique for serotyping of the clinical isolates, serotype a *A. actinomycetemcomitans* strain reacted to serotype a-specific antiserum and serotype b-specific antiserum equally at the concentration above 1:800 dilution, and serotype c strain reacted similarly to 3 serotype-specific antisera at higher concentration than 1: 800 dilution, suggesting existence of common antigen among 3 serotypes.

Our result revealed approximately equal distribution of serotype a, b, and c in overall patients, in contrast to previous report that juvenile periodontitis patients exhibited about two-fold higher frequency of serotype b than of serotype a and c combined, and serotype c was a rare serotype in these patients, suggesting that serotype b strain may be particularly virulent in destroying periodontal tissue (serotype selectivity).[30,66]

In this study, 3 of 9 patients harbored 2 different serotypes of *A. actinomycetemcomitans* strains in the same site or different sites, which is contrast to the previous finding that no patients had more than one serotype.[44,64,66] Williams *et al.*[60] observed that juvenile periodontitis patients had elevated serum antibody reacting with 2 serotypes or 3 serotypes, suggesting the possibility of 2 or 3 serotypes of *A. actinomycetemcomitans* infected. To elucidate the infecting route of this species, further study should be done to compare the serotype distribution before and after treatment.

Leukotoxicity appears to be a unique potential of *A. acinomycetemcomitans* and its role in the pathogenesis of LJP is conceivable by the finding that more serotype b produced leukotoxic factor than did other 2 serotypes, although it has not been firmly established.[60] According to Tsai *et al.*[58] preexisting PMNL dysfunction[21] increases the likelihood of infection by organisms, such as *A. actinomycetemcomitans,* that do not colonize the gingival crevice in otherwise normal individuals. PMNL function is further weakened by the action of leukotoxic factor which destroys significant number of crevicular PMNL and tissue macrophages. This situation encourages proliferation of *A. actinomycetemcomitans* and/or the establishment of the potential periodontopathic bacteria prior to the synthesis of antileukotoxic antibody. The prevalence of leukotoxic strains in examined isolates was 22% and the percentage of LDH release ranged from 2.5 to 10.7%. These values were two-fold lower than those of other studies, because whole viable bacteria at the ratio of 50 per PMNL were used in this study instead of using sonic extract.[2,3,56,65] The prevalence was also much lower than 55% in report by Zambon *et al.*.[66] The finding that both leukotoxic and nonleukotoxic strains occurred in the same sites is consistent with previous report.[65] A relationship between the prevalence of leukotoxic strains and the severity of disease was not established.

The observation that nonleukotoxic strains

were recovered at one point in time as the sole *A. actinomycetemcomitans* from 4 diseased sites suggests that leukotoxicity may not be essential in the pathogenesis of LJP if there's no sampling error to select nonleukotoxic strains. Secondly, it is possible that the infecting strain may have converted from a leukotoxic to a nonleukotoxic strain by leukotoxin-neutralizing serum activity.[31,58] Third explanation is that microbial behavior in subgingival domain is not equal to *in vitro* behavior. Cultivation of microorganism in a synthetic medium has been shown to result in loss of toxic activity in other bacteria and reculturing of *A. actinomycetemcomitans* isolates *in vitro* may also result in the loss of leukotoxicity.[3]

Serotype selectivity in leukotoxicity in previous report was inconsitent with the result from our study. This disparity could be explainable by the possibility that predominance of serotype b in Caucasian LJP might have large number of serotype b strains and few strains of the other serotypes tested.

Present study suggests that *A. actinomycetemcomitans* has no serotype selectivity in Korean LJP lesion and leukotoxicity may be not an essential but an important factor in the pathogenesis of LJP. It may be beneficial in the prevention and the treatment of LJP to identify the factors responsible for the loss of leukotoxicity. For further study, it will be necessary to determine the distribution of leukotoxic strains in the periods of disease progression and remission, and to relate that to serum neutralizing activity.

SUMMARY

Previous studies from our laboratory suggested that Korean LJP patients might habor *A. actinomycetemcomitans* of different serotype from Caucasian LJP patients in whom serotype b was predominant.

In order to observe the prevalence and serotype distribution of *A. actinomycetemcomitans* in localized juvenile periodontitis patients and to evaluate leukotoxic activity of oral isolates, this study was performed.

A. actinomycetemcomitans was isolated by using a selective medium (tryptic soy agar supplemented with 10% serum, 75μg of bacitracin and 5μg of vancomycin per ml). Using immunoabsorbed, ammonium sulfate-fractionated serotype-specific antisera, a total of 69 strains were serologically categorized by ELISA.

Leukotoxicity was monitored biochemically by measuring lactate dehydrogenase indicator of cell viability in culture supernatant of PMNL plus viable *A. actinomycetemcomitans* mixture.

The results were as follows:

1. *A. actinomycetemcomitans* was detected in 75% of 16 LJP patients, and 71% in the LJP lesions and 6% in the control sites.
2. Presence or absence of *A. actinomycetemcomitans* in the sampled disease sites has no influence on clinical measurements.
3. Three serotypes were approximately equally distributed in overall 9 patients. Three patients harbored 2 different serotypes of *A. actinomycetemcomitans* in the same disease site or different disease sites.
4. The proportion of leukotoxic oral isolates was 22% of a total of 46 strains and the prevalence was 69% in 13 sampled sites.
   The same disease site could harbor both leukotoxic and nonleukotoxic strains.
5. Distribution of leukotoxic strains in 3 serotypes were not different.

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