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Presence of Anti-oral Streptococcal Antibody in the Sera of Rheumatoid Arthritis Patients

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In this study, the antibody titers to surface antigens of several oral streptococcal strains that are known to have antigen similar to SR protein were compared in 48 rheumatoid arthritis (RA) patients and 40 healthy people (control). Antibody titers to surface antigens of oral streptococci were determined by enzyme-linked immunosorbent assay (ELISA) using whole cells, sonicated cells, or sonic extracts of *S. mutans* OMZ 175, *S. sobrinus* 6715, *S. rattus* FA-1, *S. rattus* BHT, *S. cricetus* E49, *S. gordonii* DL1 and *S. gordonii* M5. ELISA was carried out using the sera extensively absorbed with *S. rattus* FA-1 that is known to have no surface antigen cross-reactive with SR protein. Although, overall antibody titers of RA patients to mutans group streptococci (*S. mutans* OMZ 175, *S. sobrinus* 6715, and *S. cricetus* E49) were higher compared to control, difference in antibody titer between the RA patients and control group was not significant. Interestingly, however, the average antibody titer of RA patients' sera for non-mutans group *S. gordonii* DL1 and M5 was found to be significantly higher compared to control ($p < 0.001$ and $p < 0.0001$, respectively). Furthermore, in reverse of their antibody titers for mutans group streptococci, the antibody titers were greater in RF-negative sera than in RF-positive sera, although the difference was significant only for M5 ($p < 0.05$). We found that the reactive surface antigen on *S. gordonii* M5 that is related to the high titers of RF-negative sera remained with the cell after sonication of the bacteria. Sonic extracts gave more or less similar reactivity to all sera in ELISA and no specific band could be detected in immunoblot assay with high-titer sera compared to low-titer sera. The significance of this finding remains to be seen.

Key words : rheumatoid arthritis, rheumatoid factor, mutans group streptococcus, *Streptococcus gordonii*

Introduction

In oral cavity, many types of streptococci exist and these bacteria are known to share common surface antigens with others (Schöller *et al.*, 1981). Many of these antigens are known to be involved in bacterial adherence to tissue surfaces (Appelbaum and Rosan, 1984). While several investigators reported that some of these streptococcal cell surface antigens may cross-react with human tissues (Russell, 1987; Lehner *et al.*, 1991), it was reported that *S. mutans* SR protein and human IgG share cross-reactive antigenic determinant (Wachsman *et al.* 1989) and that rheumatoid factor (RF), an autoantibody which react to multiple epitopes on the Fc portion of the IgG molecule, cross-reacted with *S. mutans* (serotype f) SR protein (Ackermans *et al.*, 1991). SR protein is a 115-135 kDa saliva-interacting surface protein of *S. mutans* (Ogier *et al.*, 1984; Ackermans *et al.*, 1985), whose gene and expression product have been cloned and studied in detail (Sommer *et al.*, 1987; Ogier *et al.*, 1989). Subsequently, Gangloff *et al.* (1992) were able to identify SR protein and human IgG cross-reactive determinants by epitope mapping using recombinant proteins and synthetic peptide.

RF, generally defined as autoantibodies, are present in >70% of patients with rheumatoid arthritis (RA) and, therefore, is highly characteristic of the disease (Tighe and Carson, 1997). Substantial evidence favors the view that RF contributes to tissue injury in RA. Therefore, better understanding of RF production would help elucidate the pathogenesis of RA. Because it has been suggested that streptococci may play a role in the pathogenesis of several kinds of autoimmune diseases, it would be interesting to know if any of

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those antigens is involved in the RF production.

In this study, antibody titers of Korean RA patients to several oral streptococcal strains were studied, to better understand the relationship between the oral streptococci and RA pathogenesis.

Materials and Methods

Sera

Sera of 48 RA patients were obtained from Seoul National University Hospital, Seoul, Korea, where all the patients were previously diagnosed of RA on the diagnostic criteria established by American College of Rheumatology. The control sera were obtained from 40 healthy people during their routine health check-up. The sera had been stored in -20°C freezer. We examined the presence of RF in patients' and control sera by commercial rapid slide test (RapiTex RF, Behring Diagnostics Inc, MA, USA). Human IgG-coated latex beads were mixed well with 40 μl of undiluted serum on the test plate, and the presence of RF was determined by the degree of agglutination of latex beads according to the criteria given by the manufacturer.

Preparation of bacterial cells and sonic extracts

Mutans group streptococci, *S. mutans* OMZ 175 (serotype f), *S. sobrinus* 6715 (serotype g), *S. cricetus* E49 (serotype a), *S. rattus* FA-1 and BHT (serotype b), and non-mutans streptococci, *S. gordonii* DL1 and M5 were used in the study. Bacteria were culture in Brain Heart Infusion (Difco, Michigan, USA) for 18 hrs at 37°C aerobically in CO_2 incubator (5% CO_2). Bacterial cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and sodium azide. After adjusting to 3.0×10^9 cells/ml using a spectrophotometer (Spectronic 21D, Bausch & Lomb, USA), bacterial cell suspensions were stored in the 4°C refrigerator until use.

To obtain the sonic extracts of *S. gordonii* M5 and DL1 strains, the bacterial cells (600 mg wet weight) were resuspended in distilled water and sonicated for 3 to 5 minutes (Sonic Dismembrator Model 300, Fisher Scientific Co., USA). After centrifugation bacterial cells and supernatants were collected separately. The sonic extracts were adjusted to 5 μg/ml and immediately used to coat the ELISA plate or stored at -20°C. The cells were stored at 4°C until use, as mentioned above.

Pre-absorption of sera

Sera were absorbed as described previously (Tart and van de Rijn, 1993) with *S. rattus* FA-1 that is known to contain no cross-reacting antigen similar to SR (Moisset *et al.*, 1994). *S. rattus* FA-1 was cultured and washed as described above and the bacterial cells (0.15 g each) were distributed to microcentrifuge tubes. After 1 ml of diluted sera (1:50 with PBS) were added to each tube, bacterial cells were suspended well in the diluted serum, and the tubes were rotated on a rotating wheel for 2.5 hrs at 4°C. The pre-absorbed sera were centrifuged at 12,000 rpm for 5 min to remove the bacterial cells, transferred to a new tube, and stored with sodium azide (0.1%) at 4°C.

Enzyme-linked immunosorbent assay (ELISA)

Bacterial cells or the sonic extracts were immobilized onto wells of microtiter plates (96-well, flat bottom, Corning, USA). The bacterial cells were washed in PBS one time, resuspended in carbonate buffer (0.15 M, pH 9.6, with 0.02% sodium azide) to a density of 1.0×10^9 cells/ml, and dispensed 100 μl per well. The sonic extracts were adjusted to 5 μg/ml and dispensed 50 μl per well. The plates were stored at 4°C for 24 hrs and subsequently used in the assay. Just before assay, each wells were washed three times with 150 μl of PBS containing 0.05% Tween 20 (PBST) and blocked for 1 hr with gelatin (0.5%) in PBST.

Absorbed serum was diluted to 1:200 with PBST and 50 μl of diluted serum was added to the bacterial cell- or sonic extract-coated microtiter plate, and incubated for 1 hr at room temperature. After the wells of the 96-well plate were washed three times with 150 μl of PBST, 50 μl of 1:10,000 diluted peroxidase conjugated goat anti-human IgG (H+L chain specific, Pierce, USA) was added and incubated further for 1 hr. After washing, 50 μl of 2,2'-azino-bis[3-ethylebenzthiazoline-6-sulphonic acid] diammonium salt (ABST, Pierce, Illinois, USA) were added and incubated for 30 min for color development. Absorbance was read at 405 nm (Titertek Multiskan Plus MKII, Flow Lab, Finland).

Analysis of bacterial proteins

Sonic extracts of *S. gordonii* M5 was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, USA) for 1 hr at 60-100 volt (Mini-Protean, Bio-Rad, USA). The separated

proteins were transferred to PVDF membrane (Immobilon-P, Millipore, USA) by electroblotting for 1 hr at 100 volt (Novex, USA). After the membrane was blocked in Tris-buffered saline (TBS) containing 5% non-fat milk, absorbed serum diluted to 1:50 with TBS containing 1% non-fat milk was added and incubated at room temperature for 1 hr. After washing three times with TBS, alkaline phosphatase-conjugated goat anti-human IgG diluted 1:5000 with TBS contining 1% non-fat milk was added to the blot and incubated for 30 min. After washing three times with TBS, color was developed with 5-bromo-4-chloro-3-indolylphosphate/*p*-nitroblue tetrazolium chloride (BCIP/NBT).

Data analysis

Statistical analysis of the data were done with Analyse-It® for Microsoft Excel program, version 1.30 (Analyse-It® Software Ltd, UK).

Results

The presence of RF in 33 RA patients (68.8% of the total patients) was verified by latex agglutination method. None of the control serum had RF. Accordingly, during the analysis of the experimental results, RA patients were sometimes divided into RF-positive and negative groups. The RF-positive rate of the patient group of this study was comparable to as generally acknowledged (70%). To verify if RA patients have high titer of antibody for oral streptococci, sera from RA patients and control group were examined by ELISA. RA patients were found, on the average, to have high antibody titer for several strains of oral streptococci compared with control group. However, on the contrary to our expectation, the difference was not apparent for the mutans group streptococci. Rather, highly significant difference was found in the case of antibody titers to *S. gordonii* M5 and DL1 strains (Table. 1). Overall antibody titers of all groups were highest for *S. mutans* OMZ 175.

When we further divided the patient group by RF-positivity, we could see that the differences in antibody titers for the mutans group streptococci were limited to RF-positive group and those for *S. gordonii* strains were limited to RF-negative group (Fig. 1). Antibody titers of RF-negative group for mutans group of streptococci were not different from those of normal persons.

Because this results indicate the possibility that RF-positive and negative groups may be derived from the different populations, we set about another experiment to identify the antigen on both strains of *S. gordonii* used in the experiment that correlate with significantly higher antibody titers of RF-negative patient group. First, we wanted to see if the antigen could be released by ultrasonic treatment and could be recognized by immunoblot assay. However, we could not see any difference in the pattern of immunologically stained bands between the patient group and the normal group (Fig. 2).

This result prompted us to have a look at the sonicated cells for the putative antigen. When we compared the whole cells, sonicated cells, and

Table 1. Anti-streptococcal antibody titers of RA patients and healthy persons†

		n	Mean	SD	SE	95% CI of Mean	Median	IQR	95% CI of Median
S. mutans	Normal	40	210.0	69.89	11.05	187.6 to 232.4	199.7	96.9	178.0 to 223.3
OMZ175	Patient	48	228.1	92.95	13.42	201.1 to 255.1	214.7	114.7	184.0 to 256.0
S. sobrinus	Normal	40	99.3	56.52	8.94	81.2 to 117.4	90.2	86.4	59.7 to 117.5
6715	Patient	48	128.2	94.42	13.63	100.8 to 155.7	115.7	100.2	83.0 to 130.7
S. cricetus	Normal	40	149.2	61.01	9.65	130.0 to 169.0	142.6	81.6	116.3 to 167.3
E49	Patient	48	158.6	93.76	13.53	131.3 to 185.8	139.3	68.3	123.0 to 155.7
S. rattus	Normal	40	130.4	79.28	12.54	105.0 to 155.7	113.8	132.3	70.7 to 149.0
BHT*	Patient	48	96.6	48.52	7.00	82.5 to 110.7	94.9	77.1	66.3 to 110.0
S. gordonii	Normal	40	145.1	42.10	6.66	131.6 to 158.6	143.2	72.8	124.3 to 161.3
M5**	Patient	48	191.7	63.20	9.12	173.3 to 210.0	180.4	97.5	157.4 to 214.8
DL1**	Normal	40	161.7	65.89	10.42	140.7 to 182.8	168.8	98.0	129.3 to 190.0
	Patient	48	221.0	89.12	12.86	195.1 to 246.9	217.0	134.8	166.0 to 257.3

†Determined by ELISA and expressed as optical density × 1000.
* Significant difference between normal and patient group at $p < 0.05$
** Significant difference between normal and patient group at $p < 0.01$.

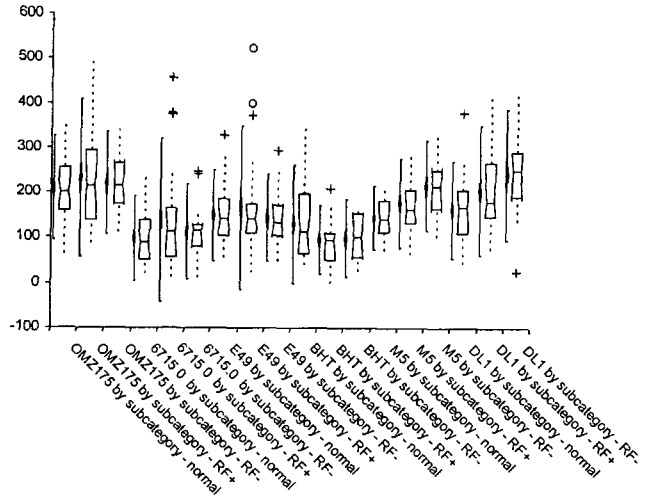

Fig. 1. The antibody titer to the surface antigens of *S. mutans* OMZ 175, *S. sobrinus* 6715, *S. cricetus* E49, *S. rattus* BHT, and *S. gordonii* M5 and DL1 in RF-positive and negative patients and normal persons. The antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using the whole cells as antigen. The sera were extensively absorbed with *S. rattus* FA-1, known to have no surface SR-like antigen. Horseradish peroxidase-labeled goat anti-human IgG was used as secondary antibody and ABTS was used as a color substrate. The differences in anti-streptococci antibody titer between patients and normal controls were analyzed by 1-way ANOVA using Analyse-It® for Microsoft Excel (ver. 1.30) program. The line series shows parametric statistics: The diamond shows the mean and the requested confidence interval around the mean. The notched lines show the requested parametric percentile range. The notched box and whiskers show non-parametric statistics: The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 IQRs (inter-quartile ranges) of the lower and upper quartiles. The crosses (+) and circles (o) indicate possible outliers - observations more than 1.5 IQRs (near outliers) and 3.0 IQRs (far outliers) from the quartiles.

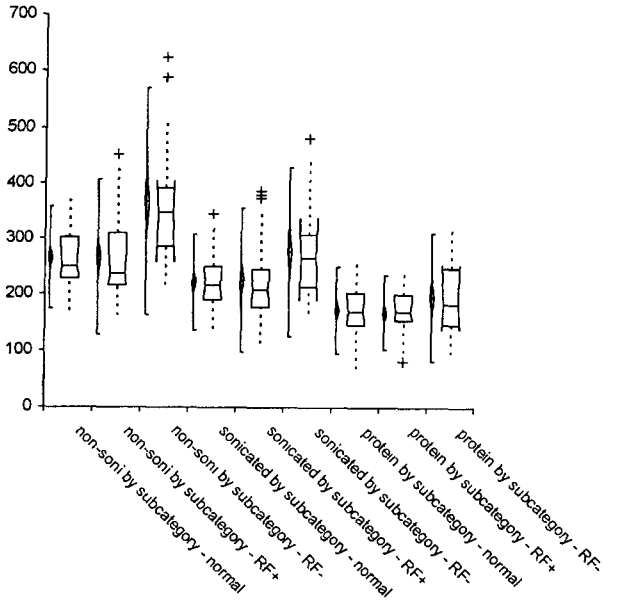

Fig. 3. The antibody titer to *S. gordonii* M5 in RF-positive and negative patients and normal control. The antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using the whole cells, sonicated cells, or sonic extract as antigen. See Fig. 1 for detail. The differences in anti-streptococci antibody titer between patients and normal controls were analyzed by 1-way ANOVA using Analyse-It® for Microsoft Excel program.

sonic exract in another round of ELISA, it was the sonicated cell, along with the whole cell, that reacted differently to RA patients' sera. The sonic extracts of *S. gordonii* M5 reacted to all sera more or less to a same degree. The difference in antiboody titers for *S. gordonii* that we saw with whole cells roughly remained with sonicated cells (Fig. 3).

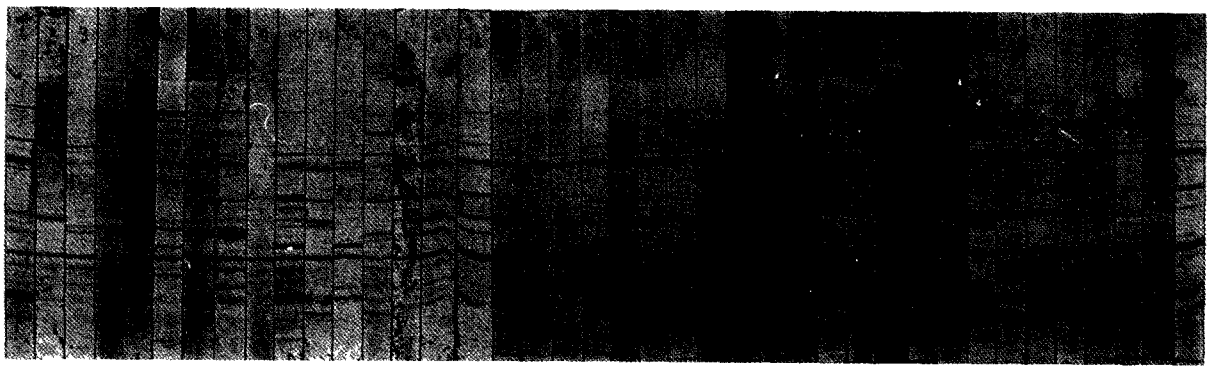

Fig. 2. Immunoblot analysis of sonic extract of *S. gordonii* M5. Sera from 8 normal control (#1-8) and 32 RA patients (#9-40) were used as primary antibody. Antibody titers were previously determined in the ELISA procedure. Alkaline phosphatase-conjugated goat anti-human IgG was used as secondary antibody and BCIP/NBT as color substrate.

Discusion

We showed that RA patients have high titers of anti-streptococcal antibody in their serum. However, further analysis indicated that this reactivity of the serum for streptococci might not be the same one as reported by Ackerman *et al.* (1989), who showed that RF cross-reacted with S. *mutans* (serotype f) SR protein. Our data did show that RA patients' sera reacted to mutans group streptococci somewhat higher than normal control group, although the results were not highly significant ($p < 0.1$ for S. *sobrinus* 6715; not statistitically significant for others). However, the reactivity of RA patients' sera was found, unexpectedly, to be highly significant for both strains of S. *gordonii* used in this study ($p < 0.0001$ and $p < 0.001$ for M5 and DL1, respectively). Furthermore, reactivity of RF-negative RA patients' sera for S. *gordonii* was higher than that of RF-positive RA patients' sera ($p < 0.05$), indicating that RF may not be responsible for this reactivity. Further study on the identity of the putative antigen on S. *gordonii* that reacted to RF-negative RA patients' sera indicated that it may be a cell wall component that could not be released by sonication. Its identity is currently unknown.

It seems clear that the reactivity of the RA sera for S. *gordonii* is not related to the presence of RF and, thus, its generation has no relation to the homology between SR protein and IgG Fc reported previously (Ackermas *et al.*, 1991; Gangloff *et al.*, 1992). However, high antibody titer in RF-negative RA patients for S. *gordonii* is a unique finding that seemed to deserve further studies. The presence of RF has been correlated with more severe disease course. Thus, for RF-negative patients, it may be argued that the pathogenic mechanism of RA might have been different from that of RF-positive patient, and that it is possible that S. *gordonii* plays a role in inducing high titer of hitherto unrecognized antibody in pathogenic way. It is known that, other than RF, autoantibodies to collagen is another marker of RA (Menzel *et al.*, 1975) with strong circumstantial evidence for a pathogenic role in RA.

No etiologic agent or primary antigen has yet been implicated in the pathogenesis of RA (Sewell and Trentham, 1993). Despite the failure of intense efforts to confirm the presence of an infectious micro-organism in rheumatoid synovium, the concept that RA is infectious in origin has continued to be attractive. Theories on the autoimmune nature of RA have benefited from the enormous progress made in understanding the cellular and molecular components of normal immune responses (Weyand and Goronzy, 1997). However, convincing experimental evidence of a joint-specific endogenous antigen in the synovial lesions is still lacking

Through a study of antigen I/II that is similar to SR, Moisset *et al.* (1994) showed that antigen I/II contains a domain that can react to salivary glycoprotein and another one that is cross-reactive to human IgG. They suggested that because these domains are well conserved and widespread among oral streptococci, oral streptococci may induce RF generation through antigenic mimicry by SR or similar salivary glycoprotein-binding protein. These antigens are variously named as SR, I/II, B, IF, P1, SpaA, PAc, MSL-1, Pag, SspA or Ssp5.

Another possibility was put forward by the presence of Fc binding protein on the surface of streptococci that binds to the same antigenic determinant as RF (Nardella *et al.*, 1985, 1987 & 1988; Sasso *et al.*, 1988; Schroder *et al.*, 1986 & 1987; Stone *et al.*, 1989). Nelson *et al.* (1985 & 1987) and Oppliger *et al.* (1987), based on their study of RF idiotype, suggested that antigenic mimicry by streptococcal Fc-binding protein through idiotype-anti-idiotype system may be involved in the generation of RF. Polysaccharide of group B streptococci (Zborovsky *et al.*, 1990), peptidoglycan of group A streptococci (Todome *et al.*, 1992), and bacterial superantigen (Goodacre *et al.*, 1994) also have been suggested to play important roles in the pathogenesis and progression of rheumatoid arthritis.

Our data showed that S. *gordonii* may be related to RA pathology, although the nature of this relation is currently unknow. This might serve as another evidence for involvement of oral streptococci in autoimmune phenomena in the body.

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