**The Periodontal Pathogen Aggregatibacter Actinomycetemcomitans Affects Experimental Autoimmune Myocarditis in Mice**

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**Summary**

Recent reports assert that dental health is linked to an increased risk of cardiovascular disease. It is well known that Aggregatibacter actinomycetemcomitans (A.a.) is highly associated with heart disease. Indeed, we previously reported that A.a. affects the development of heart disease in a mouse model. However, no reports have clarified the relationship between A.a. and experimental autoimmune myocarditis (EAM). The aim of this study was to investigate the relationship between A.a. and EAM in mice. EAM was induced via the injection of cardiac myosin into the mice. A.a. or PBS was then injected into the mice using a chamber implanted into the back of each mouse. The weight of the organs and echocardiograms were obtained and a pathological analysis and quantitative RT-PCR were performed. Echocardiography showed that no statistical difference was observed between the two groups. A histopathological analysis demonstrated that the number of areas affected by myocarditis in the A.a.-injected EAM group was significantly increased compared to that observed in the PBS-injected EAM group ($P < 0.05$). The hearts of the mice in the A.a.-injected EAM group exhibited significantly increased expressions of MMP-9 mRNA compared to the hearts of the mice in the PBS-injected EAM group ($P < 0.05$). These results show that A.a. aggravated EAM via an enhanced MMP expression. (Int Heart J 2013; 54: 412-416)

**Key words:** MMP-9, Inflammation

Myocarditis, an inflammatory heart disease, is commonly associated with viral infections and autoimmune disorders. Experimental autoimmune myocarditis (EAM) in mice induced via immunization with a specific pathogenic cardiac myosin peptide as an adjuvant has been used to investigate the pathogenesis of myocarditis. Periodontitis is known to be a cause of systemic inflammation. A previous study reported that the level of Aggregatibacter actinomycetemcomitans (A.a.) is an indicator of periodontitis activity. We also previously reported that A.a. deteriorates ventricular remodeling following myocardial infarction and transverse aortic constriction. A.a. is a major periodontopathic pathogen and is frequently detected in patients with acute coronary syndrome, and produces multiple virulence factors and tissue-damaging toxins, such as leukotoxin and an apoptosis-inducing toxin. The aim of this study was to clarify the relationship between A.a. and EAM.

**METHODS**

**Mice:** Male BALB/c A/Jc mice (7 weeks old, 20-25 g) were purchased from Japan Clea, Co. (Tokyo). The mice were given a standard diet and water maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was also approved by the Animal Care and Use Committee of Tokyo Medical and Dental University. We divided the mice into two groups: an A.a.-injected EAM group and a PBS-injected EAM group. The murine hearts were harvested 21 days after the induction of EAM.

**Mouse subcutaneous chamber model:** We used a modification of the subcutaneous chamber model, as previously described. Chambers (length: 10 mm, diameter: 5.0 mm) constructed from coils made of stainless wire were implanted subcutaneously into the back of each mouse. Two weeks later, the chambers were used as biological compartments to induce inflammation.

**Induction of EAM:** Alpha MHC peptide (Japan Bio service Co., Ac-RSLKLMATLFSTYASADR-OH) was emulsified with an equal volume of adjuvant complete Freund (Difco Lot 0097536) to a final concentration of 1.0 mg/mL. The emulsion was injected into the back of each mouse subcutaneously at a dose of 0.1 mL (0.2 mL/mouse), which yielded an immunizing...
dose of 100 µg cardiac myosin per mouse. The day of myosin infection was defined as day 0.\textsuperscript{11}

**Bacterial growth:** *Aggregatibacter actinomycetemcomitans*, strain Y4, was cultured on TSBV agar plates and then incubated in 5% CO\textsubscript{2} in air at 37°C for 3 to 5 days. The bacterial concentrations were standardized to 10\textsuperscript{5} colony forming units (CFUs)/mL, and the subcutaneous injections were performed once a week for 3 weeks starting from day 0. Figure 1 shows the time schedule of this study.

**Organ weight:** We measured the body weight and weight of the heart in each mouse 21 days after the induction of EAM and calculated cardiac weight per body weight.

**Echocardiograms:** We performed transthoracic echocardiography 21 days after immunization. An echocardiogram with a 7.5-MHz transducer (Toshiba, Tokyo) was used for echocardiography recording. We obtained the echocardiograms under anesthesia (3.6% trichloro acetaldehyde monohydrate). M-mode echocardiograms were obtained at the papillary muscle level, and the ejection fraction (EF), fractional shortening (FS) and diastolic interventricular septum thickness (IVSTd) were calculated.

**Histopathology:** The hearts were harvested immediately after the mice were sacrificed. We obtained 3 transverse sections per heart for the histological examination. The slices were stained with hematoxylin and eosin (HE). The areas of myocardium affected by cell infiltration were classified as infiltrated areas. We measured the number of affected areas using a computer-assisted analyzer (Scion Image Beta 4.0.2). The affected area ratio (affected/entire area expressed as a percentage) was calculated as previously described.\textsuperscript{11,12}

**RNA isolation and RT-PCR:** Total RNA was isolated from each individual heart using the TRIzol reagent (Invitrogen/Life Technologies, San Diego, CA) following homogenization with a polytron homogenizer. Real-time PCR was used to determine the mRNA expressions of MCP-1 (Assay ID: Mm00441242_m1), IL-6 (Assay ID: Mm00446190_m1), IL-10 (Assay ID: Mm00439616_m1), MMP-9 (Assay ID: Mm000600163_m1) and IFN-gamma (Assay ID: Mm00801778_m1) in the hearts. The cDNA was run in duplicate. To account for differences in cDNA preparation and cDNA amplification efficiency, the mRNA expression of each of the target genes was normalized according to that of 18S rRNA (4308329). The quantitative data were calculated using the comparative CT (ΔΔCT) method.

**Immunohistochemistry:** Frozen sections were incubated with primary antibodies against CD4, CD8, and CD11b (monoclonal antibodies from PharMingen, San Diego, CA) for 24 hours at 4°C. Incubation with a Histofine Simplestain Kit as a secondary antibody (Nichirei Corporation, Tokyo) was carried out at room temperature for 30 minutes. Cell numbers were determined by counting all the fields.

**Statistical analyses:** All data are expressed as the mean ± SE. Differences in the data between the two groups were analyzed using the unpaired Student’s \( t \)-test. \( P < 0.05 \) was considered to indicate statistical significance.

**RESULTS**

**Cardiac weight/body weight ratio:** The cardiac weights were comparable between the A.a.-injected EAM group and the PBS-injected EAM group 21 days after EAM induction. The cardiac per body weight ratios were also comparable between the A.a.-injected EAM group and the PBS injected EAM group (Figure 2).

**Echocardiograms:** We examined cardiac function using

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**Figure 1.** Time schedule. A.a. or PBS injection was performed on days 0, 7 and 14. Cardiac myosin peptide injection was performed on days 0 and 7.

**Figure 2.** Cardiac weight/body weight ratio. The cardiac weights and cardiac weight/body weight ratios were comparable between the two groups.
echocardiograms. There were no statistically significant differences in cardiac function or morphology between the A.a.-injected EAM group and the PBS-injected EAM group (Figure 3).

**Histopathology:** The hearts in the A.a.-injected EAM group exhibited severe inflammatory cell infiltration. In contrast, moderate inflammatory lesions were observed in the hearts in the PBS-injected EAM group. The number of affected areas was greater in the A.a.-injected EAM group than in the PBS-injected EAM group (Figure 4).

**RT-PCR:** We examined the levels of MCP-1, IL-6, IL-10, MMP-9, and IFN-gamma mRNA using quantitative RT-PCR. The MMP-9 mRNA levels were significantly increased in the A.a.-injected EAM group compared to those observed in the PBS-injected EAM group ($P < 0.05$). The MCP-1, IL-6, and IFN-gamma levels were increased in the A.a.-injected EAM group compared to those observed in the PBS-injected EAM group. The IL-10 mRNA levels tended to be lower in the A.a.-injected EAM group than in the PBS-injected EAM group. However, there were no statistically significant differences in the MCP-1, IL-6, IL-10, and IFN-gamma mRNA levels between the two groups (Figure 5).

![Figure 3. Echocardiograms. Representative M-mode echocardiograms of animals in the PBS-injected EAM group (A) and A.a.-injected EAM group (B) at 21 days. Quantitative data for EF (C), FS (D) and IVS (E) are demonstrated. There were no statistically significant differences between the two groups.](image1)

![Figure 4. Histopathology. Representative cross-sections of the hearts with hematoxylin and eosin staining of myocardial lesions on day 21. The original magnification in panels A and B was × 20. The scale bar is 1 mm. The dotted lines indicate affected areas. The original magnification in panels C and D was × 400. The scale bar is 10 µm. The hearts in the PBS-injected EAM group (A and C) and the A.a.-injected EAM group (B and D) exhibited inflammatory cell infiltration. The number of affected areas was greater in the A.a.-injected EAM group than in the PBS-injected EAM group (E). (*$P < 0.05$)](image2)
Figure 5. Quantitative RT-PCR. Representative results of quantitative RT-PCR. The MMP-9 mRNA levels were significantly increased in the A.a.-injected EAM group compared to those observed in the PBS-injected EAM group. The cardiac MCP-1, IL-6, IL-10, and IFN-gamma mRNA levels were comparable between the A.a.-injected EAM group and the PBS-injected EAM group. (*P < 0.05)

Figure 6. Immunohistochemistry. Representative findings of CD4 (A), CD8 (B) and CD11b (C) staining are shown. The arrows indicate positive cells. CD4 and CD11b-positive cells significantly increased in the A.a.-injected EAM group compared to the PBS-injected EAM group (panels a, b and c in A and C) (*P < 0.05). CD8 positive cells were comparable between the A.a.-injected and the PBS-injected EAM group (panels a, b and c in B). Original magnification of all panels was × 400. Scale bars = 10 µm.
Immunohistochemical findings: CD4, CD8, and CD11b positive infiltrating cells were detected in both groups. We counted CD4, CD8, and CD11b positive infiltrating cells. The number of infiltrating CD4 and CD11b-positive cells was significantly higher in the A.a.-injected EAM group compared to the PBS-injected EAM group (Figure 6A and Figure 6C). The CD8 positive infiltrating cells were comparable between the A.a.-injected EAM group and the PBS-injected EAM group (Figure 6B).

**DISCUSSION**

Periodontitis is one of the most common infectious diseases in humans. Periodontal inflammation plays a role in the initiation and progression of cardiovascular disease. Myocarditis is caused by large amounts of inflammatory cells migrating into the myocardium. Previous investigations have shown that the production of proinflammatory cytokines and MMPs affects the development of EAM. Periodontal bacteria generate host immunological inflammatory responses, which stimulate the secretion of cytokines and MMPs. MMPs play an important role in cell migration and are involved in the migratory capabilities of inflammatory cells, such as T-cells. In this study, we detected inflammatory cell infiltration in the hearts of mice in both groups. It has been reported that the prevalence of A.a. is higher in patients with acute coronary syndrome (ACS) than in those with chronic coronary heart disease (CHD). ACS patients exhibit significantly higher serum IgG titers against A.a. than patients with chronic CHD. The results of the present study suggest that A.a. influences systemic inflammation, which worsens cardiac inflammation. Our experiments showed that MMP-9 mRNA expression was enhanced in the A.a.-injected EAM group. We assumed that the MMP-9 mRNA expression promotes an inflammatory response. It is reasonable to conclude that the injections of A.a. significantly increased the MMP-9 mRNA levels.

Periodontopathic bacteria present in biofilms can act as reservoirs for medically important pathogens that cause systemic disorders. A.a. produces multiple virulence factors and tissue-damaging toxins, such as leukotoxin and apoptosis-inducing toxin. A.a.-lipopolysaccharide (LPS) is a well-known potent inducer of pro-inflammatory mediators. LPS derived from A.a. stimulates different receptor complexes to induce inflammatory cytokines. LPS has been shown to be a major ligand for Toll-like receptor (TLR)-4 signaling.

This study has a limitation. An echocardiogram on day 21 did not show a difference between the two groups during the entire time-course of the investigation. Further investigation is needed to elucidate how inflammation occurs and complicates cardiac function during the entire time-course of myocarditis.

In conclusion, infection with a specific periodontal bacterium is a potential risk factor for the development of acute myocarditis.

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**REFERENCES**