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Original Article

HEAT SHOCK PROTEIN OF *MYCOPLASMA SALIVARIUM* AND *MYCOPLASMA ORALE* STRAINS ISOLATED FROM HIV-SEROPOSITIVE PATIENTS

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Abstract

It has been suggested that infection by some mycoplasma species can act as possible cofactors in the acceleration of immunodeficiency in HIV-infected patients. The present study was designed to examine infections by oral mycoplasma species in HIV-seropositive (HIV(+)) patients. *Mycoplasma salivarium* and *Mycoplasma orale* were isolated from 59.5% and 16.7% of 42 HIV(+) patients, respectively. Non-*M. salivarium* and non-*M. orale* species were isolated from 40.5% of saliva samples from the HIV(+) group and 20.8% of those from 24 HIV-seronegative (HIV(-)) subjects, respectively. Although the production of superantigen by human peripheral lymphocytes in the isolated mycoplasma species from HIV(+) and HIV(-) subjects was evaluated, none of the examined mycoplasma strains, including ATCC strains of *M. salivarium*, *M. orale*, *Mycoplasma buccae* and *Mycoplasma penetrans*, were found to produce superantigen. Production of heat shock proteins (HSPs) by isolated mycoplasma strains was examined by immunoblotting using monoclonal antibodies against *Helicobacter pylori* HSP60. It was found that all the strains of *M. salivarium*, *M. orale*, and unidentified mycoplasma species isolated from HIV(+) and HIV(-) groups produced heat shock proteins. HSP production by oral mycoplasma may play a role in the immunomodulation of HIV(+) patients.

Key words: Heat shock protein—HIV-positive—Oral mycoplasma species

INTRODUCTION

It has been suggested that infections by mycoplasma species are possible cofactors in

accelerating the development of AIDS in HIV-infected patients^{4,7,19}. Certain mycoplasmas have been shown to reinforce the cytotoxicity of HIV^{4,14-16}. *Mycoplasma fermentans*,

Table 1 Population, age, clinical status of the 42 HIV-infected subjects and 24 HIV-noninfected control subjects examined in this study

Study population	HIV(+) group (n = 42)	HIV(-) group (n = 24)
Mean age	35.5	32.5
(Range)	21–58	23–57
Men/Women	38/4	19/5
Hemophiliac	38.1%	ND*
Heterosexual	19.0%	ND
MSM	42.9%	ND

*: Not determined

Mycoplasma penetrans, *Mycoplasma genitalis*, and *Mycoplasma pirum* have been isolated from AIDS patients^{5,9,11}. Analysis of mycoplasma species in urine from HIV-positive children indicated that AIDS-associated mycoplasma infections were more common in HIV-infected children than in HIV-negative controls¹¹. *Mycoplasma salivarium* and *Mycoplasma orale* have been shown to be members of the human oral microbial flora^{10,17,23,24}. However, few studies have searched for mycoplasma species in the oral cavities of HIV-seropositive (HIV(+)) subjects. Both the pathogenicity of these mycoplasma species and their relationship with the development of AIDS remains unclear.

It is known that microbial infections can modulate host responses. Production of superantigens and of heat shock proteins (HSPs) is closely associated with changing host responses^{1–3,6–8,21,22,26,27}. This study was designed to evaluate the significance of infections by *M. salivarium* and *M. orale* in HIV(+) patients. We also examined the production of superantigens and HSPs by *M. salivarium* and *M. orale* strains isolated from HIV(+) subjects.

MATERIALS AND METHODS

1. HIV(+) and HIV(+) subjects

A total of 42 HIV(+) subjects attending the Department of Internal Medicine at Juntendo University Hospital, the AIDS Clinical Center at the International Medical Center of Japan, or the Dental Clinic at the Kanagawa Children's

Medical Center were recruited for the study. The population of this study is summarized in Table 1. The HIV(+) group consisted of 38.1% hemophiliacs, 19.0% heterosexuals, and 42.9% homosexual individuals. Twenty-four HIV(-) volunteers at Tokyo Dental College were recruited as the control group for this study.

After obtaining informed consent, saliva samples were collected from all the subjects. They were instructed to rinse with a solution containing 5 ml of phosphate-buffered saline (PBS, pH 7.2) for 15 sec. and then spit the saliva mixture into sterile plastic tubes. One hundred microliters of each saliva sample were cultured in order to examine them for the presence of mycoplasma.

2. Detection and identification of mycoplasma species

To detect and quantify the mycoplasma species, mycoplasma agar plates (BBL Microbiology System) containing 10% fresh baker's yeast extract, 10% inactivated rabbit serum and 20 IU/ml penicillin G were used as described in our previous study⁵. Inoculated plates were incubated in an anaerobic chamber for two weeks. Colonies grown on these agar plates were repeatedly inoculated on fresh culture plates and then cultured in mycoplasma broth for three days. After purification, grown cells in the mycoplasma broth were examined by the polymerase chain reaction (PCR) targeting 16S rRNA locus as described by Blanchard *et al.*⁴. The fragments of 16S rRNA locus in the cells of isolates were also sequenced to identify

the species.

For the identification of isolated mycoplasma, PCR was performed with a Mycoplasma Detection Kit (Takara Shuzo, Tokyo) according to the manufacturer's instructions. Briefly, PCR was performed by adding 2 μ l of sample to 98 μ l of reaction mixture containing 10 μ l of 10 \times PCR buffer, 8 μ l of 200 μ M dNTP, and 0.25 U Taq polymerase. The sequences of the primers are as follows, F1: ACACCATGGGAGCTGGTAAT, R1: CTTTCATCGACTTTCAGACCCAAGGCAT, F2: GTTCTTTGAAAACCTGAAT, R2: GCATCCACCAAAAACCTCT. PCR was performed for 30 cycles at 95°C for 30 sec, 55°C for one min, and 72°C for one min. Elimination of primers from amplified products was performed by SUPREC 02 (Takara Shuzo). The resulting product was sequenced with a Big Dye Terminator Sequence Kit (Perkin Elmer, San Diego, CA.). The DNA homology search was performed with the Blast network service in DDBJ.

3. Assay for superantigen production

The cultured supernatant of the isolated mycoplasma strains was examined for the production of superantigen. Strains of *M. salivarium* ATCC 23064, *M. orale* ATCC 23714, *Mycoplasma buccae* ATCC 14851, and *Mycoplasma penetrans* ATCC 14854 were included in this experiment. Cells grown in mycoplasma broth were filtered through a 0.1 μ m Sterile Millex-VV filter (Millipore Co.) and evaluated for superantigen activity.

To obtain lymphocytes, heparinized venous blood (30 to 50 ml) of healthy donors was diluted with PBS, and layered over Ficoll-Conray containing 100 g of Ficoll 400 (Pharmacia) and 240 ml of Conray 400 (66.8w/v %, Daiichi Seiyaku, Tokyo) per liter. The density of this solution had been adjusted to 1.077 with saline. The layered tubes were centrifuged for 30 min. at 2,600 rpm at room temperature. Leukocytes were harvested from the Ficoll-Conray interface and washed twice with Hank's solution (Nissui, Tokyo) containing 2% fetal calf serum to remove Ficoll. Cells were collected by centrifugation at 1,500 rpm

for 10 min. at 4°C and then resuspended in the RPMI 1640 culture medium with antibiotics supplementation of 100 μ g/ml streptomycin and 100 IU/ml penicillin. For the superantigen assay, 1 \times 10⁵ viable cells per well were distributed in 96 well round bottom microplates (Iwaki, Chiba, Japan).

Staphylococcus enterotoxin A (SEA; Toxin Technology Inc., Gainesville, FL) was used to stimulate lymphocyte culture as a positive control at a concentration of 400 ng/ml. For the assay, either 4 μ l of supernatant bacteria cultured broth, the appropriate concentration of SEA as a positive control, or only broth as a negative control was added to the cells. After incubation at 37°C in humidified environment containing 5% CO₂ for 2 days, the cultures were pulsed with methyl-³H thymidine (1 μ Ci/well) for the last 16 hours of incubation, and thymidine incorporation was determined in a liquid scintillation counter. The data are presented as the average of triplicate cultures.

4. Production of HSP

To detect HSP antigens, monoclonal antibodies against *Helicobacter pylori* HSP 60 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used²⁵. Grown cells of isolated mycoplasma strains including *M. salivarium* ATCC 23060 and *M. orale* ATCC 23714 were also examined in this study.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 to 20% gradient Micro Slab Gels (Daiichi Pure Chemical Co., Tokyo). Cells grown in mycoplasma broth were centrifuged, and pellets were obtained. Pellets were washed with PBS and spun for 10 min. The resultant pellets were resuspended in PBS and 30 μ l were mixed with 10 μ l of SDS-PAGE sample buffer. After boiling the samples, each one was loaded onto an SDS-PAGE gel and transferred to a PVDF membrane (Immobilon, Millipore, Bedford, Ma.) using trans-Blot (BIO-RAD Laboratories, Hercules, CA) as described by Towbin *et al.*²⁰. After transferring, the PVDF membranes were blocked with 3% BSA in PBS at 37°C for 60 min and incubated

in 1:1,000 diluted anti-*Helicobacter pylori* HSP60 antibody (Wako, Osaka, Japan) for 60 min as a first antibody. After washing, peroxidase-labeled goat anti-rabbit IgG immunoglobulins (BIO-RAD Laboratories) were used at a 1:3,000 dilution as secondary antibodies. Development was performed with 10 mM Tris-HCl buffer (pH 7.5) containing 0.04% of 4-methoxy-1-naphthol, 10% ethanol, 0.9% NaCl, and 0.08% of H₂O₂.

RESULTS

1. Detection rates of mycoplasma species

Mycoplasma were detected by the PCR method in 25 in 42 HIV(+) subjects (59.5%) and in 16 in 24 control subjects (66.7%). A comparison of the amplified sequences at the 16S rRNA locus from isolated mycoplasma showed that *M. salivarium* was present at the same frequency in the two groups (16.7%). The *M. orale* detection rate by PCR was 2.4% in HIV(+) subjects, while that in HIV(-) was 33.3%. This difference was significant ($p < 0.01$). Rates of unidentified mycoplasma species in isolates from HIV(+) subjects were 40.5%, and those in HIV(-) subjects were 20.8%. Thus, the colonization of unidentified mycoplasma species in HIV(+) patients was significantly higher than those in HIV(-) subjects ($p < 0.05$).

2. Superantigen activity of culture supernatant by isolated mycoplasmas

Superantigen activity in cultured supernatants of 21 strains isolated from saliva samples from HIV(+) and HIV(-) subjects and ATCC strains of *M. salivarium*, *M. orale*, *M. buccae* and *M. penetrans* were evaluated. None of the cultured supernatants from 8 *M. salivarium*, 6 *M. orale*, and 7 unidentified mycoplasma strains showed strong proliferating activity of lymphocytes in two or more of the samples obtained from three volunteers. The results suggested that none of the mycoplasma strains isolated from the oral cavity of HIV(+) subjects produced superantigen.

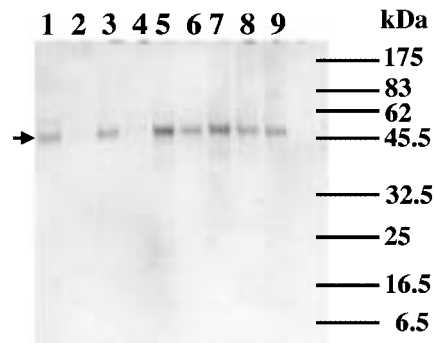


Fig. 1 Immunoblotting results of mycoplasma strains isolated from the oral cavity of HIV-seropositive patients. All strains formed clear bands with the monoclonal antibody against *Helicobacter pylori* HSP60.

3. Production of HSP by isolated mycoplasma

It was found that all the strains of *M. salivarium*, *M. orale*, and unidentified mycoplasma isolated from the saliva samples of HIV(+) patients produced HSPs that reacted with the monoclonal antibody against *H. pylori* HSP 60. Some of the western blotting results in isolated *M. salivarium*, *M. orale* and unidentified mycoplasma species isolated from HIV(+) patients are shown in Fig. 1.

DISCUSSION

We examined the PCR detection rates of *M. salivarium* and *M. orale* species in saliva samples from an HIV(+) group of 42 subjects and compared them with those in an HIV(-) group of 24 subjects. The mycoplasma detection rates were similar in the two groups. *M. orale* detection rates in control subjects were significantly higher than those in HIV(+) subjects. In our previous study, we reported that the prevalence of mycoplasma species in 67 HIV(+) subjects was statistically lower than that in 32 HIV(-) subjects. However, in the present study, we found that the detection rates of mycoplasma species that were neither *M. salivarium* nor *M. orale* were significantly higher in saliva samples from HIV(+) subjects than in those from the con-

trol group ($p < 0.05$). Future study protocols have been developed in order to identify the mycoplasma species isolated from HIV(+) subjects that were neither *M. salivarium* nor *M. orale*.

Immunomodulatory effects are known to be implicated in the progression of immunodeficiency HIV patients. There are various immunomodulating factors produced by microorganisms, including superantigen and HSPs. Many research groups have suggested that infections by mycoplasma species are cofactors in the seroconversion of AIDS in the HIV(+) group^{7,9,11,14,18,19,24}. As one such cofactor, superantigen produced by specific mycoplasma species is known to play a role in the immunodeficiency process in HIV(+) patients^{4,9,11,15,16}. However, in this study, we found that the strains of *M. salivarium*, *M. orale* and unidentified mycoplasma species we examined did not produce superantigen. Specifically, our results indicated that *M. salivarium* and *M. orale* could not produce superantigen.

HSPs are highly conserved immunogenic proteins that are immunodominant antigens produced by bacteria and host cells in response to a variety of stressors^{26,27}. They have been implicated in immunomodulatory actions such as immunosuppression and induction of autoimmune diseases^{6,26,27}. The present study showed that mycoplasma species isolated from saliva samples obtained from HIV(+) and HIV(-) subjects produced HSPs. We previously reported that various periodontal disease-associated bacteria produced HSPs that cross-reacted^{12,13}. In infectious disease processes, interactions with the immune systems such as production of HSP and superantigen may be involved in immune suppression in compromised hosts. The results from the present study do not provide sufficient data to enable us to discuss the possible relationship between mycoplasma infections in oral cavity and progression to AIDS in HIV(+) subjects. However, it is possible that HSPs produced by microorganisms, including mycoplasmas in periodontal regions, form immunocomplexes that may activate the complement system and lead to inflammation of

the gingival tissues in HIV(+) patients.

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