The Pyrogenicity in Rabbits Elicited by Aqueous–Phenol Extract from Oral Mycoplasma Cells

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Abstract: Aqueous–phenol extract from *Mycoplasma salivarium* ATCC 23064 cells (APS) lacks 2–keto–3–deoxyoctonate found in almost all lipopolysaccharides (LPS) of gram–negative bacteria. We examined this APS in pyrogenicity test system of the rabbit. Intravenous application of 134 μg/kg APS to rabbit was characterized by mono–phasic fever after a short delay of 30 min. Increase in Tre (℃) was 1.10±0.18 (n=7), decrease in Tea (℃) and RR (min^{-1}) were 3.94±1.19 (n=7) and 113±78 (n=7), respectively. The pyrogenicity activity of APS was relatively strong, but the dosage so far used was too high due to impurities of APS. However, the present thermoregulatory responses indicated that APS used in this experiment contains very effective new exogenous pyrogen which is different from LPS.

Key words: Mycoplasma salivarium, aqueous–phenol extract, pyrogenicity test, fibrile response, temperature regulation

INTRODUCTION

Oral mycoplasmas are identified as *Mycoplasma salivarium* and *Mycoplasma orale* which are members of the microbial flora of gingival sulci. Watanabe *et al.* (1972) reported on the incidence or isolation of oral mycoplasmas associated with periodontal diseases. Furthermore, the high antibody response to *M. salivarium* in patients with

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various oral diseases was reported by Watanabe and Totsuka (1986). Gingival tissue is highly susceptible to endotoxin derived from plaque bacteria (Rizzo and Mergenhargen, 1964; Taichiman and Courant, 1965). Endotoxic lipopolysaccharides (LPS) have been discussed as a possible pathogenetic factor in periodontal disease (Mergenhargen *et al.*, 1961; Simon *et al.*, 1972). However, the biological activities of aqueous−phenol extract from oral mycoplasma (APS), except the several mycoplasmas and acholeplasmas (Seid *et al.*, 1980), have not been investigated especially compared with those of the gram−negative bacterial LPS. Therefore, we examined the APS in pyrogenicity test system of the rabbit as one of the biological activities. After the rabbits were injected with APS, febrile responses such as the increase of rectal temperature, decrease of ear skin temperature as well as respiratory rate were investingated in the present experiment.

Materials and Methods

The organisms used in this study were *Mycoplasma salivarium* ATCC 23064. The mycoplasma was cultivated in a liquid medium consisting of PPLO broth (Difco), 1% (w/v); yeast extract (Difco), 1% (w/v); L−arginine−HCl, 1% (w/v); 0.2% phenol red, 10ml; phosphate buffered saline, pH7.0 (PBS), 965 ml; horse serum, 2.5% (v/v). The final pH was adjusted to 7.0 by using 1 N hydrochloric acid. The usual batch of organisms consisted of 60 liters of a 72 hours culture started from a 1% inoculum. Incubation was carried out at 37℃ in 2−liter volumes contained in 2−liter flasks. Growth was assessed by a change in indicator color to an alkaline pH. The organisms were harvested by centrifugation, followed by sedimentation at 21,000 ×g for 30 min at 4℃. The sediment was washed three times in PBS and was washed three times in sterilized pyrogen−free distilled water, and lyophilized.

Freeze−dried organisms were extracted three times with chloroform−methanol (2:1, v/v) (1 g of cell dried per 30 ml of chloroform−methanol) to remove free−lipids. The dried residues after lipid extraction were stirred with 45% aqueous−phenol (1 g of residues per 35 ml of aqueous−phenol) at 65℃ to 68℃ for 15 min (Westphal *et al.*, 1952). The aqueous layer was collected after centrifugation at 12,000 ×g for 30 min at 4℃. The phenol layer was reextracted with an equal volume of distilled water. The combined aqueous layers were dialyzed for 72 hours at room temperature against several changes of distilled water. After the dialysis, the extract was centrifuged at 100,000 ×g for 2 hours at 4℃. The sediment was washed four times with the same volume of distilled water to remeve nucleic acid. After the lyophilization, the fluffy white material was dissolved (2 mg per 1 ml) in sterilized isotonic saline (Ohtsuka Co., Tokushima, Japan) for the pyrogenic reactions in rabbits. The suspension was sonicated at 160 watts for 4 min at 4℃ by using a sonicator (Heat Systems−Urtrasonic, Inc. USA). All water and saline used were pyrogen−freed in this experiment.

Rabbit pyrogenicity tests were performed on four male albino rabbits weighing from 2.5 kg to 3.0 kg. These animals were pyrogen negative and had no signs of infec-

tious diseases. The experiments were carried out on the animals restrained in conventional rabbit box, at ambient temperature and humidity of 28℃ and 60%, respectively. One animal was used as a control. Rectral temperature (Tre,℃) and ear skin temperature (Tea,℃), were measured continously using thermister probes (Ellab Co., USA), and respiratory rate (RR,min^{-1}) was monitored by recording impedance changes in electrolyte−filled distensible rubber tube placed around the animal's thorax (Ohwatari and Kosaka, 1979). To test the febrile response to pyrogen, 134 μg of APS per kg was injected intravenously, and 30 mg of antipyretic sulpyrine (Daiichi seiyaku, Tokyo) per kg was also administered. Precautions were taken to eliminate the probability of pyrogen contamination; injection cannula, syringes etc. were sterilized by heat at 250℃ for 2 hours before use. The pyrogenic responses induced by APS in rabbit were expressed either as the net increase in Tre or the net decrease in Tea and RR. The statistical analysis of pyrogenic responses was performed mean value and standard deviation of pyrogenic responses in each animals was statistically performed.

Results and Discussion

APS could be extracted into the aqueous phase of hot 45% phenol. The preparation showed varying solubility when suspended in distilled water or isotonic saline, whereas ultrasonic treatment at maximum output rendered highly dispersive suspensions. The quantitative chemical analyses, gave a recovery of 49% of the dry weight of APS, because moist was not removed completely from the preparation. Aqueous−phenol extract which was used in this study contained the following components; carbohydrate accounted for about 40% of the dry weight for APS, while hexosamine was very little. The ratios of glucosamine to galactosamine were about 2:1 in APS. Glycerol and phosphorus accouted for 0.24% and 2.09%, respectively. APS was devoid of 2−keto−3−deoxyoctonate, alanine, cystein, methionine and tyrosine, but contained aspartic acid, glutamic acid, glycine, valine. The purity of our preparation is not absolute since contaminating nucleic acids and possibly some protein could be detected (Totsuka *et al.*, 1986) (to be published). Therefore, APS could be considered as crude preparation on the results described above.

Figure 1 shows the schematic representation of fever−curves after administration of 134 μg/kg the APS. After the short latency of 30 min, mono−phasic fever curve was developed and accompanied with two falling phases of Tea and RR. The febrile state continued for over four hours after injection. When Tre was highest, 30 mg of antipyretic sulpyrine per kg was injected intravenously. Tre steeply decreased. And when Ter was lowest, the same dose of the APS was injected intravenously again. Tre become higher than that of the first injection. Mono−phasic fever−curves were elicited by 134 μg/kg of APS. These results were summarized in Table 1. Increase in Tre (℃) was 1.10±0.18, decrease in Tea (℃) and RR (min^{-1}) were 3.94±1.19 and 113±78, respectively.

It has recently been shown that the LPS would cause either mono−phasic or

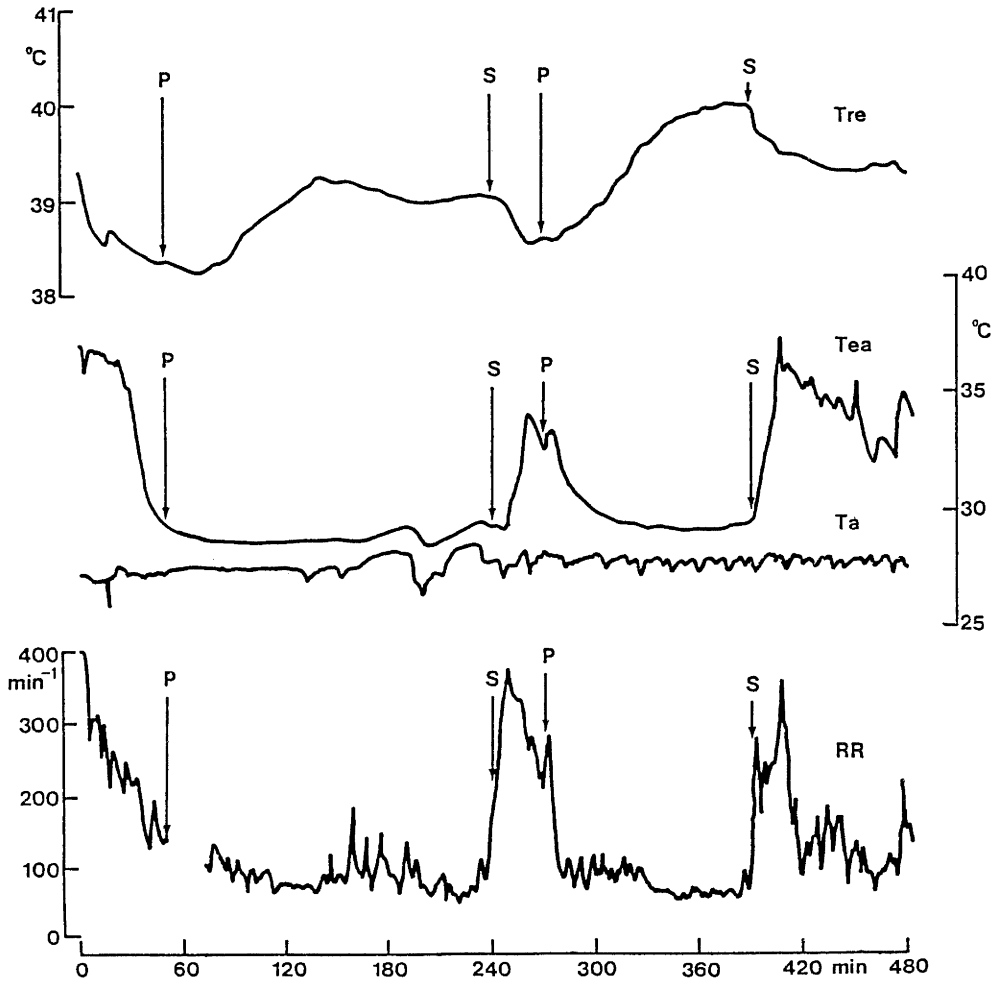

Fig. 1 Typical responses to the aqueous−phenol extract from *Mycoplasma salivarium* cells.
P; intravenous injection of the preparation (134 μg/kg wt.)
S; intravenous injection of sulpyrine (30 mg/kg wt.)
Tre; rectal temperature (℃)
Tea; ear skin temperature (℃)
Ta; ambient temperature (℃)
RR; respiratory rate (min^{-1})

Table 1. Pyrogenic reaction induced by aqueous-phenol extact from *Mycoplasma salivarium* ATCC 23064 (APS) in rabbits

Preparation	Increase in rectal temperature (°C)	Decrease in ear skin temperature (°C)	Decrease in respiratory rote (min^{-1})
APS	1.10 ± 0.18 (n=7)	3.94 ± 1.19 (n=7)	113 ± 78 (n=7)

Dose : 134 μg/ kg. rabbits

bi−phasic fever depending on the doasge. It was suggested that mono−phasic fever curve developed after intravenous administration of excess mycoplasmal pyrogen, such as lipoglycan. Lipoglycans from several species of *Acholeplasma* and from *Thermoplasma acidophilum* were examinated for endotoxin−like activities measured by the standard rabbit fever test. The lipoglycans from several species of *Acholeplasma* caused a febrile response at concentrations of 1 ng/ml per kg or greater, whereas with control *Escherichia coli* EC−2 lipopolysaccharides, 6.25 ng/ml per kg was required (Seid *et al.*, 1980).

Although the pyrogenicity activity of APS was relatively strong, the dosage so far used was too high due to impurities of APS, compared to another pyrogenic substances, therefore, it is difficult to comment on the right dose which induces mono−phasic or bi−phasic fever curve of this APS. However, APS used in our experiment contains very effective new exogenous pyrogen which is different from LPS. In order to clarify the pyrogenecity of this APS, purification must be done and different ways of administration to animals should be performed in the process of thermoregulatory investigation, in the near future.

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口腔マイコプラズマ細胞のフェノール抽出物により誘発される発熱反応

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グラム陰性桿菌のリポ多糖体 (LPS) と 2−keto−3−deoxyoctonate を欠くことで化学組成の異なる *Mycoplasma salivarium* 細胞の温フェノール水抽出物 (APS) の発熱反応をウサギで調べた．ウサギに体重1 kgあたり 134 μg の標品を耳介皮膚血管から静脈投与すると 30 分の短い潜時ののち，一峰性の発熱が観察された．直腸温度 (℃) は 1.10±0.18 (n=7) 増加し，逆に，耳介皮膚温度 (℃) および呼吸数 (min^{-1}) はそれぞれ 3.94±1.19 (n=7) および 113±78 (n=7) と減少した．これらの成績は，APS が発熱性の物質であることを示唆している．

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