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Erythromycin treatment suppresses myocardial injury in autoimmune myocarditis in rats via suppression of superoxide production

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ABSTRACT

Background: Recent evidence suggests that erythromycin (EM), a major macrolide antibiotic, has many biological functions in addition to the anti-bacterial actions, including anti-inflammatory and free radical scavenging actions. However, the effects of the drug upon inflammatory myocardial diseases are unknown. We tested the hypothesis that EM ameliorates experimental autoimmune myocarditis in rats attributing to the suppression of superoxide production.
Methods: We administered EM, 3.3 mg/kg/day and 33 mg/kg/day, intraperitoneally for 3 weeks, to rats with experimental autoimmune myocarditis (EAM) produced by immunization by porcine myosin.
Results: EM treatment reduced the severity of myocarditis compared with the untreated group in a dose-dependent manner by comparing the heart weight/body weight ratio, pathologic scores, and myocardial macrophage, $CD4^+$, and $CD8^+$ infiltrations. Echocardiographic study showed that increased left ventricular posterior wall thickness produced by myocardial inflammation was reduced by EM treatment. Myocardial superoxide production, determined by dihydroethidium staining, was significantly reduced by the treatment. Western blotting showed that the expression of myocardial interleukin-1β was reduced by EM treatment compared with untreated groups. The in vivo dorsal air pouch model showed that EM significantly suppressed leukocyte chemotaxis in a dose-dependent manner.
Conclusion: Irrespective of a well-known classic antibiotic, EM attenuated EAM not only by the anti-inflammatory action but by the suppression of superoxide production.

1. Introduction

In humans, acute myocarditis is a potentially lethal disease, and frequently precedes the development of dilated cardiomyopathy (DCM). In general, two major mechanisms to explain how myocarditis develops into DCM have been proposed; one is a persistent antigen existence, and the other is a progressive autoimmune myocardial injury [1]. The autoimmune giant cell myocarditis in rats, i.e., experimental autoimmune myocarditis (EAM), mimics human fulminant myocarditis in the acute phase [2].

Erythromycin (EM) is known as a classic 14-member ring macrolide and a potent antibiotic for the treatment of various microbial infections [3]. Recently, macrolides, including EM, have been reported to have multiple biologic effects, such as alteration of inflammatory and free radical actions [4–6]. It was demonstrated that free radicals play an important role in the development of heart failure in this animal model [7]. However, the effects of EM upon inflammatory myocardial diseases are still unknown.

The purpose of the present study was to examine the effects of EM upon an EAM model, focusing on its inhibitory effects on the production of inflammatory cytokines and free radicals.

2. Materials and methods

2.1. Immunization

EAM was induced in Lewis rats by immunization with porcine cardiac myosin as previously described [8–10]. Porcine cardiac myosin (Sigma) was injected subcutaneously in the foot pads with 0.1 ml of myosin (10 mg/ml) mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Difco) on days 1 and 8. Control rats were immunized with FCA alone. The animals were daily observed up to the end of the experiment on day 21. The day of injection was designated day 1.

Rats surviving 21 days after myosin or FCA immunization were used for the cardiac function study, and thereafter were sacrificed.

2.2. Medication

Rats with EAM were divided into three groups and treated intraperitoneally with phosphate buffered saline (PBS) (untreated group, n = 15) and EM (3.3 mg/kg/day, n = 15 and 33 mg/kg/day, n = 10) for 3 weeks (immunized protocol). These doses

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were chosen because of the previous studies [4–6]. In parallel with the protocol, additional control groups (unimmunized protocol) were age-matched unimmunized rats treated for 21 days with PBS (untreated group, n = 5) and EM (3.3 mg/kg/day, n = 5 and 33 mg/kg/day, n = 5). All the mice were sacrificed 3 weeks thereafter. Organ weights were determined. Organs including the skins at dorsal air pouch were processed for the pathologic study. The animals were sacrificed under the light ether anesthesia at the end of experiments.

We performed animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and these were approved by the institutional ethics committee for animal experiments of Kyoto University.

2.3. Histopathology

At sacrifice, macroscopic findings were performed. Microscopic findings for myocardial necrosis and cellular infiltration were graded on a scale of 0 to +5, as previously described [8–10]. That is, myocardial sections were graded by two of the authors (K.S., C.K.) who had no knowledge of the respective treatment groups on a scale of 1+ (mild) to 5+ (very severe) for the severity [8–10]. After macroscopic examination, a part of the ventricles was embedded in OCT for immunohistochemistry.

2.4. Immunohistochemical staining

We used an immunoperoxidase technique to perform immunohistochemistry for cell surface markers, as previously described [8–10]. In brief, anti-macrophage (anti-Mϕ, ED1, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), and anti-CD8 (53–6.7, 1:50, PharMingen) antibodies were applied to acetone-fixed cryosections. After being washed, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated antibodies), and the antibody was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. The positive-staining cells of the heart tissue were counted blindly by two observers in six fields at ×400 magnification (within a 1-mm^2 grid), and the total positive-staining cells of the six fields were recorded as the number of infiltrating cells in the lesions. The percentage of stained cells was then calculated for each antibody staining section [8,11].

2.5. In situ detection of superoxide production in hearts

To evaluate in situ superoxide production from hearts, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [11–14]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from hearts, fluorescence (intensity x area) was measured in five randomly selected fields using the high-power image monitor.

2.6. Western blotting

Western blot analysis for interleukin-1β (IL-1β) was performed as described previously [7,9,10]. The myocardial lysates were electrophoresed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were sequentially electrophoretically transferred to a membrane (Millipore). The membrane was incubated with an anti-IL-1β antibody (Serotec) and then with a peroxidase-linked secondary antibody (Amersham). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) samples were probed in the blots as internal controls for loading. Resulting bands were quantified as optical density band area by the image analysis system.

2.7. Echocardiographic and hemodynamic studies

Echocardiography was performed on day 21 post-myosin injection for the subpopulation of control (immunized and unimmunized) and EM (33 mg/kg/day, immunized and unimmunized) groups. Rats were anesthetized with an intraperitoneal injection of 0.040 to 0.060 mg/mg sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips). Left ventricular (LV) end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), interventricular septum (IVS) thickness, and posterior wall (PW) thickness were measured, as an average of three beats. Ejection fraction (EF) of the left ventricle was calculated [10,11].

2.8. Chemotaxis in vivo

A dorsal air pouch was created as previously described [15]. Briefly, rats were injected on the back subcutaneously with 4 ml of air under the anesthesia of sodium pentobarbital on day 10. PBS (0.3 ml) was injected into the air pouch. Air pouch was maintained up to day 20. The air pouch was flushed quantitatively with PBS, and 4 h later, the volume recovered was measured. The number of cells was determined with a hemocytometer. The exudating cell numbers into the air pouch on day 20 were calculated. The cutaneous tissues at the dorsal air pouch were pathologically examined.

2.9. Statistical analysis

All values were expressed as means ± standard deviation (SD). One way analysis of variance (ANOVA), followed by the Fisher protected least significant difference test, was performed. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. General clinical findings

No rats died in all groups during the three week study.

Table 1
Organ weights and cardiac pathology.

		(n)	BW	HW/BW	LuW/BW	LiW/BW	Cardiac pathology (0 to +5)	
			(g)	(×10^{-3})	(×10^{-3})	(×10^{-3})	Infiltration	Necrosis
Immunized protocol								
Control		15	261 ± 20	4.5 ± 0.4	4.3 ± 0.3	42.3 ± 4.0	3.2 ± 0.5	2.8 ± 0.7
			(249,271)	(4.3,4.7)	(4.1,4.5)	(40.1,44.5)	(2.9,3.5)	(2.4,3.2)
Erythromycin	3.3 mg/kg/day	15	270 ± 17	4.3 ± 0.5	4.1 ± 0.5	42.2 ± 3.8	2.7 ± 0.9	2.5 ± 0.9
			(261,279)	(4.0,4.6)	(3.8,4.4)	(40.1,44.3)	(2.2,3.2)	(2.0,3.0)
	33 mg/kg/day	10	258 ± 10	3.8 ± 0.4*	4.1 ± 0.3	41.6 ± 2.8	1.8 ± 0.7*	1.9 ± 0.4*
			(251,265)	(3.2,4.1)	(4.0,4.2)	(39.6,43.6)	(1.3,2.3)	(1.6,2.2)
Unimmunized protocol								
Control		5	260 ± 30	3.8 ± 0.2	4.3 ± 0.3	42.0 ± 4.0	–	–
			(223,297)	(3.6,4.0)	(3.9,4.7)	(37.0,47.0)		
Erythromycin	3.3 mg/kg/day	5	258 ± 25	3.7 ± 0.4	4.4 ± 0.4	43.4 ± 3.8	–	–
			(228,288)	(3.2,4.2)	(3.9,4.9)	(38.7,48.1)		
	33 mg/kg/day	5	238 ± 21	3.9 ± 0.2	4.0 ± 0.3	39.0 ± 4.2	–	–
			(212,264)	(3.7,4.1)	(3.6,4.4)	(33.8,44.2)		

(mean ± SD)

HW/BW ratio was lower in the erythromycin group (33 mg/kg/day) compared to the control. Semi-quantitative histological grades for infiltration and necrosis were significantly lower in the erythromycin group (33 mg/kg/day) compared to the control. BW = body weight, HW = heart weight, LuW = lung weight, LiW = liver weight, Parenthesis indicates 95% confidence interval (CI).

* $P<0.05$ vs control.