

An animal model of chronic rheumatic valvulitis induced by formalin-killed streptococci

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Received: 24 June 2009 / Accepted: 27 November 2009 / Published online: 12 December 2009
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Abstract Rheumatic heart disease is the most severe complication of rheumatic fever. Till date, very few successful animal models of rheumatic valvular disease have been reported. This study aimed at developing a suitable animal model of chronic rheumatic valvulitis for further investigation and prevention of rheumatic heart disease. Lewis rats were immunized with one administration of formalin-killed and sonicated group A streptococci together with Complete Freund's Adjuvant every 7 days for three cycles followed by group A streptococci alone till killing. Control rats were administered adjuvants and saline. Rats in group 1 were killed 12 weeks after the initial injection.

Rats in group 2 and control group were killed 24 weeks after the initial injection. Results 62.5% (5/8) of rats in group 1 developed myocarditis and 50% (4/8) developed valvulitis. Histological examination of cardiac sections showed only cellular infiltrates. In contrast, 75% (6/8) of rats in group 2 developed rheumatic-like myocarditis and 62.5% (5/8) developed chronic valvulitis. Histological manifestations of the hearts in group 2 animals involved not only acute damage such as cellular infiltrates, Aschoff-like cells, verrucous vegetation, but also chronic lesions such as fibrosis, vascular neogenesis. None of the rats (0/8) in control group presented myocarditis or valvulitis. Lewis rat repeatedly immunized with formalin-killed GAS may be a suitable animal model of chronic rheumatic valvulitis. It may be useful for future investigation of the pathogenesis and possible preventive strategies of human rheumatic heart disease.

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Keywords Group A streptococcus · Lewis rat ·
Rheumatic valvulitis · Rheumatic heart disease

Introduction

Rheumatic fever (RF) is a delayed inflammatory sequel to infection of group A streptococci (GAS). Valvular involvement in RF may result in permanent stenosis, insufficiency, or both, i.e., rheumatic heart disease (RHD), which is responsible for most of the cardiac morbidity and mortality. Although the exact pathogenesis of RHD is not very clear, there is no question concerning the crucial role of GAS in RHD. It is commonly accepted that molecular mimicry between GAS components and host heart tissue leads to a breakdown in immunological tolerance to auto-antigens. For example, streptococcal M proteins and group A

carbohydrate immunologically mimic heart antigens, such as cardiac myosin and laminin [1, 2]. Establishment of an animal model of RHD with pathological features resembling those of human RHD has been a challenge for many years. To our knowledge, animal models for the study of rheumatic valvular heart disease are very limited and none of the proposed models displays pathology similar to that seen in humans [3]. In a previous study, we confirmed the feasibility of inducing rheumatic myocarditis lesions in rats by immunization with formalin-killed GAS [4]. In this study, we tried to induce experimental model of chronic rheumatic valvulitis by injecting formalin-killed GAS to Lewis rats.

Materials and methods

Bacterial culture, inactivation and antigen preparation:

Group A β -hemolytic streptococci were grown in 3-l batches of brain–heart infusion broth (BHI-D, Bionerieux, France) with continuous stirring at 37°C for 24 h. The bacterial cells were collected by centrifugation (3,500 rpm, 20 min, 37°C), washed by resuspension and centrifugation three times with sterile normal saline. The sediments were immersed in 50 ml of 10% formalin solution for 6 h, washed five times, and suspended in sterile normal saline. The formalin-treated streptococci were inoculated on sheep blood plates. No bacterial growth was observed after 24 h of culture. From 3 l BHI-D culture and after formalin treatment, the wet weight of the harvested bacterial cells was about 7 g. The absorbance at 520 nm of the final bacterial suspension was adjusted to 2.5 OD. Then, the suspended cells were stored on ice, disrupted by sonic vibration (8 w \times 10 min/10 ml), then emulsified in Complete Freund's Adjuvant (CFA, Sigma, USA) at a 1:1 ratio (v/v).

Animals

The experimental protocol was performed in accordance with ethical guidelines stated in Guide for the Care and Use of Laboratory Animals. Experiments were performed on 7-week-old female Lewis rats that weighed 150–190 g (provided by Vital River Laboratory Animal Technology Co. Ltd, Beijing).

Induction of valvulitis

Animals were randomly divided into three groups (eight rats per group). On day zero, 0.2 ml mixture of sonicated GAS and CFA was injected into the rear foot pad of groups 1 and 2 animals, followed by a subcutaneous administration of 0.5 ml mixture of sonicated GAS and CFA on day 7 and

14. Control animals received sterile normal saline emulsified in CFA. After the three treatments, group 1 and 2 animals received subcutaneous injection of 0.5 ml sonicated GAS solution without CFA every 7 days, and control group was subcutaneously injected with saline till killing.

Twelve weeks after the initial immunization, animals in group 1 were killed by anesthesia with 5% chloral hydrate injected intraperitoneally. Rats in group 2 and control group were killed 24 weeks after the first injection by same anesthesia.

Laboratory tests

Serum anti-streptolysin O antibody (ASO) tests were performed by latex agglutination technique using 50 μ l of rat's sera and one drop of reacting solution containing ASO-sensitized latex particles. Anti-deoxyribonuclease B (anti-DNAse B) tests were performed by microtitremetry. Anti-DNAse B assay kit used was obtained from Guangdong Institute of Cardiovascular Diseases (Guangzhou, China).

Histological examination of cardiac tissue

Rat hearts were extracted and fixed in 10% neutral buffered formalin overnight prior to being embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin for light microscopic histological examination.

Results

Laboratory tests

Serum ASO and anti-DNAse B titers of all animals were negative.

Histological examination of cardiac tissue

Examination of myocardial sections showed that focal cellular infiltration in the interstitial areas near the vessel was present in 62.5% (5/8) of group 1 and 75% (6/8) of group 2 animals (Table 1). The enlargement of myocarditis in group 2 showed the presence of Aschoff-like cells near the small vessels, a characteristic of rheumatic carditis (Fig. 1a). They look like “caterpillars” in longitudinal section and like “owl-eye” cells in transverse section. Vasculitis was also observed in group 2 as characterized by the presence of mononuclear cell in vessel wall and endothelium hyperplasia (data not shown). In contrast, none in group 1 displayed Aschoff-like cells.

Because of their small size, it was difficult to see all of the valves of the rat heart. Mitral valve could be seen in all

Table 1 Myocarditis and valvulitis in Lewis rats immunized with formalin-killed GAS

Group	No. of rats with/no. of tested	
	Myocarditis	Valvulitis
Group 1 ^a	5/8	4/8
Group 2 ^b	6/8	5/8
Control ^c	0/8	0/8

^a Immunized with formalin-killed GAS emulsified in CFA and killed on week 12

^b Immunized with formalin-killed GAS emulsified in CFA and killed on week 24

^c Immunized with CFA and saline, and killed on week 24

rats. Valvulitis were seen in 50% (4/8) of group 1 and in 5 of 8 62.5% (5/8) of group 2 (Table 1) hearts, but there was much difference between the two groups. Valvular examination of group 1 only displayed cellular infiltration (Fig. 2a, b). The most interesting observation was the valve lesions in group 2 which involved cellular infiltrates composed primarily of lymphocytes, macrophages and multinucleated giant cells, valvular thickening, swelling, verrucous vegetation along closing edge of valve, vascular neogenesis (Fig. 2c, d). Acute and chronic pathological manifestations co-existed in the same rat in group 2.

Myocardial and valvular structure of control group was normal. None of the rats in control group had myocarditis and/or valvulitis (Figs. 1b, 2e, f).

Discussion

There was no doubt about the close relationship between GAS and RF/RHD. GAS comprises capsule, cell wall, and cell membrane. Capsules of the streptococci used in our study were not apparent. After treated with formalin and sonic, the bacteria became inactive and were not able to produce exotoxin and extracellular enzyme, but antigen in cell wall and cell membrane was preserved. Streptolysin O and streptodonaase (also called streptococcal deoxyribonu-

lease) are extracellular enzymes secreted by live streptococcus [5]. The bacteria in our study were dead and could not produce streptolysin O and streptodonaase, so hosts should not react to these enzymes. This explains why serum ASO and anti-DNAse B of all animals in group 1 and group 2 were negative. This is in accordance with rheumatic carditis patients whose serum ASO and anti-DNAse B were negative but the histological examination of heart revealed active inflammation.

It is well known that the processes of pathology of RF are divided into three stages: exudation, proliferation, and sclerosis. When streptococcal infections are recurrent, these changes may co-exist in the same host. The characteristic of acute stage is the presence of Aschoff's nodules. They ultimately "heal" by fibrosis. At chronic stage, endocarditis heals by progressive fibrosis, leaflets become thickened and neovascularization. The pathological repairs of valve leaflets convert a translucent, pliable valve into a stiff, thickened structure which is responsible for the most important long-term sequel of rheumatic fever, and usually are clinically manifested decades after the acute process [5].

In recent decade, there were some researches to induce acute rheumatic valvulitis, but few experimental lesions have met the standards of typical Aschoff's nodules [6–8]. Cunningham MW work group reported an animal model of valvular heart disease. They used streptococcal recombinant M6 protein as an antigen to induce myocarditis and valvulitis in Lewis rats. Histopathological examination of cardiac tissue 17 days after the primary immunization showed that 50% of the rats (3/6) developed myocarditis and valvulitis. Valvulitis was characterized as cellular infiltrates, verruca-like nodule on the valve surface. Anitschkow cells and cells with caterpillar nuclei were observed [6]. They also injected different epitopes of cardiac myosin to immunize Lewis rats. After 21 days, 43% (10/23) of Lewis rats immunized with human cardiac myosin and 46% (6/13) of rats given rat cardiac myosin developed valvulitis. Features of valvulitis included mononuclear cellular infiltrates [7]. Maybe because of the short period of induction, only acute lesions occurred, fibrosis and neovascularization were not present. Although animal models supported the

Fig. 1 Histological features of myocarditis. **a** Group 2 Aschoff-like cells near vessel, like caterpillar. **b** Control group normal myocardial tissue (H&E, ×400)

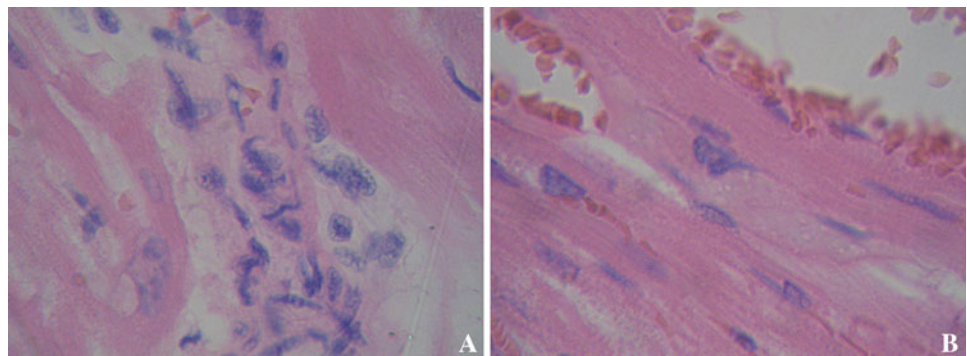
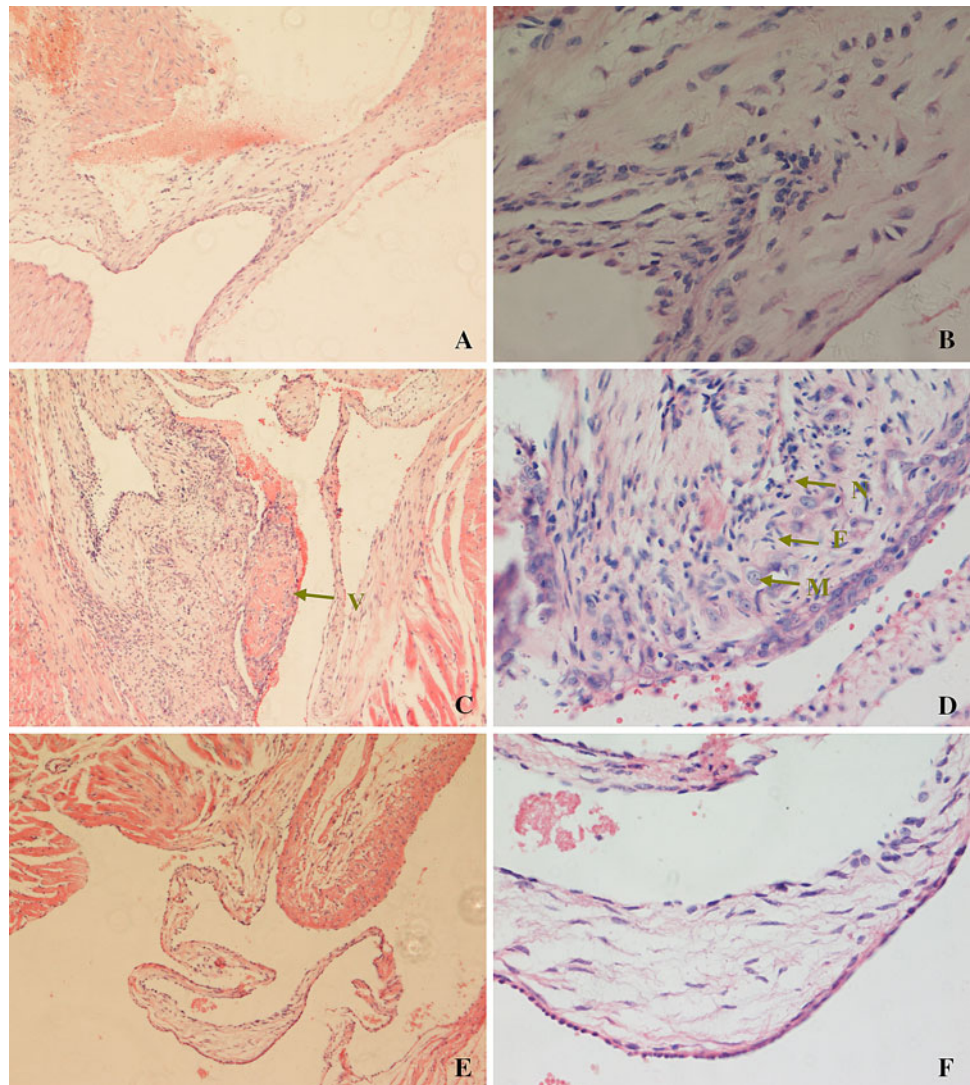


Fig. 2 Valvulitis in mitral valves from Lewis rats immunized with formalin-killed GAS. **a** and **b** *Group 1* mononuclear cells infiltrate of valve. **c** *Group 2* verrucous vegetation on the surface of valve, valve thickening, swelling, vascular neogenesis, and cellular infiltrates. **d** *Group 2* cellular infiltrates composed primarily of lymphocytes, macrophages, and multinucleated giant cells. **e** and **f** *Control group* normal structure of valve of. *V* verrucous vegetation; *M* multinucleated giant cell; *N* neovascularization; *F* valvular fibroblast [H&E, $\times 100$ (left), $\times 400$ (right)]



significant role of M protein in inducing rheumatic carditis, other pathogenic factors should also be considered.

In our experiments, components in cell wall and cell membrane such as protein, polysaccharide, and peptidoglycan were included as antigens. Based on our previous work, we confirmed the feasibility of inducing rheumatic myocarditis lesions by immunization with formalin-killed GAS again and reproduced lesions of acute exudation. In present work, the most important observation was the existence of both acute and chronic manifestations of mitral valves in the same animal in group 2. But this was not seen in group 1 animals. The only difference between two groups was the duration of induction; it was 12 weeks in group 1 and 24 weeks in group 2. The results supported that besides category of antigen, duration of induction was a critical factor. Our data suggested 12 weeks was not long enough to cause chronic valvular damage. To our knowledge, animal model of chronic valvulitis has not been previously described. Fibrosis and neovascularization are indicators of chronic

inflammation. Angiogenesis was found to be unequivocally co-dependent on chronic inflammation, which is known to involve proliferation, migration, and recruitment of inflammatory cells. Since the valve is an avascular tissue, it was assumed that valve would be inflamed by antibody deposition and subsequent lymphocytic infiltration. Repetitive stimulation with GAS antigens in spite of inactivation of the bacteria could result in lymphocyte infiltration through neovascularized regions in the scar tissue of the valve, causing perpetuation of the disease. Fibrosis leads to valvular dysfunction, more importantly, a number of different cardiac pathologies seem to be caused by a common fibrotic process. The factors leading to continued fibrosis and subsequent valvular disease remain incompletely defined.

The Lewis rat model of chronic valvulitis in the present work may be a powerful tool for further research and prevention of fibrosis and neovascularization of valvular heart disease. Relatively long experimental period and multiple

focal infections with GAS could be important factors causing the histological changes of chronic stage of rheumatic valvulitis.

In summary, this preliminary study suggested that Lewis rat repeatedly immunized with formalin-killed GAS for a period of 24 weeks may be a suitable animal model of chronic rheumatic valvulitis. The animal model will provide a useful tool for further investigation of pathogenesis of chronic valvulitis and development of other target for prevention of subsequent valvular dysfunction. For example, reduction of neovascularization and fibrosis might be other appropriate target for prevention of subsequent valvular dysfunction. Further experiments with larger samples and investigation of the causative molecules are warranted.

Acknowledgments We thank Professor Tingchung Wang (University of Rochester Medical Center) for critically reviewing the manuscript.

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