Induction of Myocarditis Lesions in Lewis Rats by Formalin-killed Cells of Group A Streptococcus

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The feasibility of inducing rheumatic myocarditis lesions in Lewis rats by immunization with formalin-killed group A Streptococcus was evaluated. The rats were divided into three groups. Group A was immunized by injecting formalin-killed group A Streptococcus suspended in complete Freund’s adjuvant (CFA) into the hind-foot pads and repeat immunization was given 1 week later by subcutaneous injection into the belly. The rats were then sacrificed 3 weeks after the initial immunization. In group B, the rats received the same initial immunization as group A, but the repeat immunization was carried out at 1, 2 and 3 weeks after the initial immunization and the rats were sacrificed 6 weeks after the initial immunization. Group C was a control group with the rats injected with saline/CFA and sacrificed on the same schedule as group A. Heart pathology sections showed that myocarditis lesions, focal inflammatory cell infiltration in interstitial near small vessels and valvulitis were induced in Lewis rats following immunization with formalin-killed group A Streptococcus.

KEY WORDS: Streptococcus pyogenes; Group A Streptococcus; Rat disease model; Rheumatic Myocarditis; Focal inflammatory cell infiltration; Valvulitis

Introduction

Rheumatic fever (RF) and rheumatic heart disease (RHD) remain significant causes of cardiovascular disease worldwide. Despite a documented decrease in the incidence of acute RF and a similar decrease in the prevalence of RHD in industrialized countries during the past five decades, these non-suppurative cardiovascular sequelae, which occur as a result of group A streptococcal (Streptococcus pyogenes or GAS) pharyngitis, remain medical and public health problems in both industrialized and industrializing countries. The most devastating effects are on children and young adults during their most productive years.

The pathogenic mechanism(s) responsible for RF and RHD are still incompletely defined. To date, no standard animal model for RF has been established and none of the animal models previously investigated have been proven fully adequate to study the pathogenic mechanisms of RF. In one study, Quinn et al. reported that 50% (three of six) of Lewis rats immunized with recombinant
Induction of myocarditis lesions in Lewis rats

J Huang, X Xie, Z-F Lin et al.

Type 6 streptococcal M protein developed valvulitis as well as focal lesions of myocarditis. Valvular lesions that initiated at the valve surface endothelium spread into the valve 17 days following immunization. Anitschkow cells and verruca-like lesions were also present. The study by Quinn et al. was the first to show the sensitivity of Lewis rats to valvular heart disease, suggesting a potentially useful and powerful tool with which to study rheumatic and immune-mediated valvular heart disease.

Although there has been success with this animal model, multiple factors other than streptococcal M protein must be considered in any animal model of human rheumatic myocarditis, however Bordetella pertussis cells (as an additional adjuvant) cannot be purchased in most countries, including China. The present preliminary study investigated the feasibility of inducing rheumatic myocarditis in Lewis rats by immunization with formalin-killed group A Streptococcus.

Materials and methods

ANIMALS

The study was performed using Lewis rats purchased from Vitalriver Company (Beijing, China). The animals were bred in a specific pathogen free animal laboratory at the Centre of Animal Experiments at Sun Yat-sen University under conditions of constant humidity and ventilation, with a light–dark cycle of 12 h. The study protocol was approved by the academic guidance group of the Division of Rheumatology, Third Affiliated Hospital of Sun Yat-sen University.

ANTIGEN PREPARATION

Group A Streptococcus was cultured in a heart–brain fluid infusion medium (BD Diagnostic Systems, Sparks, MD, USA). After harvesting, the group A Streptococcus was killed by adding 10% formalin. The concentration of formalin-killed group A Streptococcus was adjusted to $1.2 \times 10^{11}$ cfu/ml by the addition of normal saline, before diluting with complete Freund’s adjuvant (CFA) in a 1:1 (v/v) ratio.

IMMUNIZATION OF LEWIS RATS

Lewis rats that were 7 weeks old and had a body weight ranging from 152 to 173 g were divided into three groups. Group A ($n = 8$ rats) was immunized in the hind-foot pad with 0.2 ml of formalin-killed group A Streptococcus suspended in CFA. After 1 week, repeat immunization was carried out by subcutaneous injection into the belly. Group A rats were sacrificed 3 weeks after the initial immunization. Group B rats ($n = 5$) underwent the same initial immunization as group A, although repeat immunizations were then carried out at 1, 2 and 3 weeks after the initial immunization by subcutaneous injection into the belly and the rats were sacrificed 6 weeks after the initial immunization. Group C rats (control group, $n = 4$), were immunized in the hind-foot pad with 0.2 ml of normal saline/CFA 1:1 (v/v). Repeat immunization was carried out 1 week later by subcutaneous injection into the belly and the rats were sacrificed 3 weeks after the initial immunization.

ASSESSMENTS AND SAMPLE PREPARATION

The body weight of the rats was measured before immunization, and 2 and 3 weeks after the initial immunization in groups A and C. In group B, body weight was measured before immunization, and 2, 4 and 6 weeks after the initial immunization. Heart, foot, lung and kidney tissue from sacrificed rats from the three groups were fixed in 10% buffered formalin and

176

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embedded in paraffin. Sections (5 µm) were cut and stained with haematoxylin and eosin for microscopic histological examination.

Levels of anti-myocardial antibodies were measured using an assay kit (USCN Life Science & Technology Co., Missouri City, TX, USA), according to the manufacturer’s instructions.

The occurrence of swelling and arthritis was monitored in all animals.

STATISTICAL ANALYSIS
Changes in animal body weight were expressed as mean values ± SD. Statistical analysis was performed using the t-test. Statistical calculations were carried out using SPSS® version 11.0 (SPSS Inc, Chicago, IL, USA) for Windows®.

Results
BODY WEIGHT
The pre-immunization mean body weights for the three groups of rats were: group A, 158.8 ± 8.1 g; group B, 157.2 ± 9.9 g and group C, 154.0 ± 2.5 g. There were no statistically significant differences in body weight between groups A and C prior to immunization. At 2 weeks after the initial immunization, body weight was 173.3 ± 6.7 g in group C and 159.0 ± 7.4 g in group A (t-value = 3.24, P = 0.009. At 3 weeks after the initial immunization, body weight was 195.2 ± 5.5 g in group C and 181.0 ± 5.2 g in group A (t-value = 4.37, P = 0.001). Mean body weights at weeks 2, 4 and 6 for group B were 160.2 ± 17.2 g (not significantly different compared with groups A and C), 149.8 ± 13.1 g and 168.2 ± 26.7 g. Overall, the data show that increase in body weight was significantly hampered by immunization with the formalin-killed group A streptococcal suspension.

INDUCTION OF ARTHRITIS AND HISTOPATHOLOGICAL EXAMINATION
Only mild localized swelling and arthritis occurred in the injected hind-foot of rats in the control group (group C), and there was no swelling or arthritis in the non-injected hind-foot of control rats. Histopathological examination of group C showed no inflammatory cell infiltration in the soft tissue of either the injected foot (Fig. 1A) or non-injected foot (Fig. 2A).

Moderate non-localized swelling and arthritis occurred in the non-injected hind-foot of rats in groups A and B from day 3 after the initial immunization and this
became severe from day 4 after the initial immunization. This non-localized swelling and arthritis began to alleviate after 4 weeks in group B. Histopathological examination of group A showed that inflammatory cells infiltrated the soft tissue in both the injected hind-foot (Fig. 1B) and non-injected hind-foot (Fig. 2B). Histopathological examination of group B showed similar results to those of group A.

Increasing levels of anti-myocardial IgG antibodies were detected in the serum of group A and group B animals in the second week after initial immunization.

Histopathological examination of the heart showed that focal inflammatory cell infiltration in the interstitial near small vessel was present in three of the eight rats in group A (Fig. 3) and four of the five rats in group B (Fig. 4), but was not present in the control group (group C). It was not possible to see valves in all the rats, possibly because of their small size, however valves could be seen in six of the eight rats in group A, none of which had valvulitis (not shown), and in three of the four rats in the control group (group C) of which none had valvulitis (Fig. 5). Valves could be seen in all five rats in group B and valvulitis was present in two of them 6 weeks after the initial immunization (Fig. 6).

No mucinous, fibrinoid degeneration, Aschoff body-like granulomas or rheumatic vegetative formations were found.

FIGURE 2: Histopathology (haematoxylin and eosin staining) of the soft tissue of the non-injected hind-foot pad of Lewis rats showed: (A) no infiltration of inflammatory cells in the hind-foot pad of group C (control), and (B) inflammatory cell infiltration in group A could be seen at 3 weeks. Original magnification ×100

FIGURE 3: Histopathology of the heart tissue (haematoxylin and eosin staining) showed focal inflammatory cell infiltration in the interstitial near small vessel was present in group A 3 weeks after the initial immunization of formalin-inactivated group A Streptococcus. Original magnification ×200
Discussion
Many questions remain that have significant implications for choosing suitable streptococcal vaccines to protect against rheumatic carditis in humans.\textsuperscript{5 – 7} There is no direct or conclusive evidence for a pathogenetic role of cross-reactive antibodies \textit{in vivo}, nor is there a preferred animal model of RF available for study.\textsuperscript{2} Although animal models support the important role of streptococcal M protein in inducing RF and RHD,\textsuperscript{3} multiple factors must be considered in
any animal model for rheumatic myocarditis in humans, for example the roles of group A streptococcal cell walls and peptidoglycan–polysaccharide complexes in myocarditis, arthritis and uveitis in mice and rats. 8 – 12

The sensitivity of Lewis rats to valvular heart disease has been confirmed in previous studies, 3,13,14 however, to the best of the authors’ knowledge, whether formalin-killed cells of group A Streptococcus preparations can induce RF-like myocarditis and arthritis in Lewis rats had not been previously investigated.

The present study showed that arthritis develops in Lewis rats immunized using formalin-killed group A Streptococcus. Swelling and arthritis in the injected hind-foot, as well as infiltration of the foot pad by inflammatory cells, occurred in both the treated groups in this study (groups A and B). In animals injected with formalin-killed group A Streptococcus, non-localized redness and swelling occurred in the non-injected hind-foot and histopathological examination showed inflammatory cell infiltration in the non-injected foot 3 weeks after the initial immunization. In contrast, no similar non-localized inflammation or inflammatory cell infiltration was observed in the control group (group C). These results suggest that the inflammation in the treated animals (groups A and B) was possibly related to stimulation of an immune response by group A Streptococcus antigen.

More importantly, myocarditis developed in Lewis rats immunized with formalin-killed group A Streptococcus. In group A, where immunization was repeated once, inflammatory cell infiltration in the interstitial near small vessel was present but no valvulitis was observed in the heart. In contrast, in group B, where immunization was repeated multiple times, more lesions showing focal inflammatory cell infiltration were observed and valvulitis was present in two of the five rats 6 weeks after the initial immunization.

In conclusion, in the group that received multiple repeat immunizations with formalin-inactivated group A Streptococcus, 80% developed myocarditis and 40% developed valvular lesions, while no histological changes to the heart were observed in any tissues of the control animals. This preliminary study suggests that immunizing Lewis rats with formalin-inactivated group A Streptococcus may provide a suitable animal model for RF and RHD. However, the number of animals used in this study was small, and longer observations in a larger sample are needed to develop a more typical animal model for RF.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

References


