

Therapeutic Action Research of Bacille Calmette Guerin (BCG) on a Systemic Lupus Erythematosus Mouse Model

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Abstract

Background: We have been determined that for patients with systemic lupus erythematosus (SLE), after becoming infected with tuberculosis, the damages in their hematologic and complement systems were alleviated to a certain extent, this was an interesting phenomenon. So we hypothesized that tuberculosis infection could be useful for treating the SLE activity.

Methods: In order to verify the above research conclusions, the animal experiments were conducted in combination with an SLE model mouse with BCG vaccinations.

Results: The research results showed that, for the mice receiving BCG vaccinations, the serum protein complement C4 concentration was significantly higher than that of the normal saline control group. Also, the kidney damages of the experimental mice were alleviated, and their lifetimes were significantly extended.

Conclusions: The research results indicated that the BCG vaccine may have therapeutic effects on systemic lupus erythematosus (SLE).

Keywords

Systemic lupus erythematosus; Bacille Calmette Guerin; Vaccination; Complement; Therapy; Immunology

Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease which involves many factors. Due to the current unknown etiology, the therapeutic effects and prognoses are poor, which seriously affects the labor abilities and life qualities of the patients involved. The use of corticosteroids and immune suppressants can alleviate the conditions of the patients. However, the long-term use of corticosteroids may cause serious side effects, such as osteoporosis, gastro duodenal ulcers, and Cushing's syndrome. Some patients are prone to showing repeated diseases during the process of their maintenance therapy.

However, an interesting phenomenon has been observed in recent clinical studies, i.e. after being infected with mycobacterium

tuberculosis, the patients with systemic lupus erythematosus (SLE) demonstrated that the disease activities of their systemic lupus erythematosus (SLE) were under control. These patients mainly displayed decreases in white blood cells, revering of platelets, complement C3 and C4, and the mitigation of skin damages. It has usually been considered that for SLE patients infected with tuberculosis, the illness will be accentuated. The question remains as to why the lupus activities become reduced instead. Subsequently, this research study carried out a retrospective analysis of the clinical data of more than 2,000 cases of SLE patients in our hospital over the span of a decade. It was determined that the hematologic system damages (including white blood cells and platelets) of the SLE amalgamative tuberculosis patients were in fact alleviated over the relatively pure SLE patients. Furthermore, the levels of complement C3 and C4 were higher than the control group. However, the IgG, IgM, and IgA of both groups showed no significant differences ($P>0.05$). The inflammatory biomarkers of the SLE patients with amalgamative tuberculosis were observed to be aggravated, such as the acceleration of erythrocyte sedimentation, and the elevation of C-reactive protein. According to the research results, after the SLE patients were infected with tuberculosis, the illness was aggravated. However, the inflammatory response was actually aggravated, while the lupus activities were alleviated. Therefore, the data have been summarized [1], and many other researchers in this field have expressed concerns and interests in the results of this study.

Due to the complicated clinical factors which influence the prognosis of this disease, animal experiments have been carried out in order to eliminate the interference factors. The tuberculosis vaccine (BCG) was used to simulate SLE mice with tuberculosis infections, and if the BCG can be confirmed to control the activities of SLE, this will be a very significant study. This study used a method of stimulating cellular immunity (BCG vaccine), which was opposite to the traditional method of immune suppression (for example, cortical hormones) in the treatment of SLE. When ideal results could be achieved, it was not only able to promote the research of the pathogenesis of systemic lupus erythematosus (SLE), but could also lead to fundamental changes in SLE treatment concepts.

The following research was carried out in this experiment:

(1) A widely-accepted SLE mice model (MRL/LPR mice) was used to study the effects of the BCG vaccine on the SLE activities, with complements C3 and C4 as the evaluation indicators of the lupus activities.

(2) The kidney disease situations of experimental mice and control groups were comparatively analyzed, in order to discuss the influence of the BCG vaccine on the pathological changes of the lupus nephritis.

(3) The differences in the survival curves between both groups were analyzed to observe whether the BCG vaccine was able to prolong the survival rate of the MRL/LPR mice.

Materials and Methods

Materials

Animals: The MRL/LPR mice were the 12th generation among the different strains of LG/J, AKR/J, C3H/D, and C57BL/6 mice,

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Received: October 19, 2016 Accepted: November 02, 2016 Published: November 07, 2016

through a series of complex hybridization. Due to the fact that the Fas recessive mutations were associated with spontaneous programmed cell death, lymphocyte proliferation genes occurred, which led to T cell proliferation. This was similar to the clinical manifestations of human lupus erythematosus (SLE), including systemic lymph node enlargement, DNA antibodies, sm antibodies, RNP antibodies, high degrees of ANA, immune complex glomerulonephritis, vasculitis, and so on. The MRL/LPR mice are one of the commonly-used SLE animal models. The MRL/LPR mice (SPF level) had a weight of 18.4 to 23.8 g, were female, and were provided for this study by the Shanghai Slack Laboratory Animal Co., Ltd. Animal Ethical approval for the study was obtained from the Sun Yat-Sen University Ethics Committee [(2012)219].

BCG (Bacille Calmette Guerin): In this study, the BCG lyophilized powder was the attenuated live bacteria of the Bacille Calmette Guerin D2 PB 302 S11 strain (the National Standard BCG Denmark Ö strain), with a specification of 50 mg, and the number of living bacterium not less than 1×10^6 / mg. This was provided for this study by the Shanghai BioRc Co., Ltd.

Reagent and instrument: The mouse complement protein 3 (C3) enzyme-linked immunoassay (ELISA) kit (48 T), and the complement protein 4 (C4) enzyme-linked immunoassay (ELISA) kit (48 T), were provided by the Shanghai Beizhuo Biotechnology Co., Ltd. The quantitative enzyme standard instrument model was DR-200 BS, which was produced by the Wuxi Hiwell Diatek Instruments Co., Ltd.

Methods

Grouping and drug delivery: According to similar weights, the mice were matched in order to randomly be divided into normal saline and BCG treatment groups, with five mice per each group. They were caged for feeding, and then the mouse auricle was punched in order to number. Beginning in the second week of the experiment, each of the mice of the BCG treatment group was injected with 0.1 ml BCG subcutaneously on its back, and each of the mice of the normal saline group was injected with 0.1 ml of saline solution subcutaneously on its back. The injections were carried out again a week later, and then once a week, for a total of four injections.

Sample blood collection: A tail scissor was used to collect blood from the mice, and a centrifuge tube was used for the centrifugal separation of the serum. Prior to the injections of BCG and physiological saline, and after two weeks of injections, the blood collections were carried out once, respectively.

Sampling and fixing: The kidneys of the mice were extracted anatomically to check the kidney specimens, and then fixed with tissue fixative for 24 hours. Then, paraffin was used to embed the slices with HE staining for optical microscopy.

Quantitative ELISA method: A quantitative ELISA method was used to detect the serum complement protein C3 and C4 concentrations in the mice.

Kidney specimen sampling, fixing, and HE staining: A supine position was adopted for the experimental animals, which was sprawled and fixed, and then the abdominal walls were cut along the belly line from the xiphoid cartilages to the anus, followed by the cutting of the abdominal wall along the last left and right frames to the spine. All of the abdominal organs were exposed in order to check the quantity and shape of peritoneal fluid, whether the peritoneum was smooth, and the viscera position was normal, for the purpose of

cleaning up the abdominal cavity organs (spleen, pancreas, stomach, and so on). Then, tweezers were used to strip the kidney fat, and to extract the kidneys in to check the kidney specimens. The specimens were fixed with tissue fluid for 24 hours, and the paraffin embedded slices were HE stained for optical microscopy.

Renal pathological assessment: In this study, by referencing the pathological classification of lupus nephritis, and the evaluations of the renal pathological changes, three main indices of each kidney's pathological slide were observed. For example, at least 30 glomerular lesions situations, renal tubules and interstitial situations, and renal vascular inflammations were evaluated. By referencing the evaluation criteria of kidney tissue lesions, the glomerular lesions of the mice were fitted in order to divide them into active and chronic lesion groups. The kidney active lesions were characterized by the following: (1) Cell proliferation; (2) Cellular infiltration; (3) Fibrinoid necrosis and nuclear burst; (4) Cellular crescent; (5) Transparent thrombus and platinum ear-pick; and (6) Renal tubular interstitial mononuclear cellular infiltration. The chronic lesions of the kidney tissues were characterized by the following: (1) Glomerular sclerosis; (2) Fibrous crescent; (3) Interstitial fibrosis; and (4) Renal tubular atrophy.

Statistical treatment

The two groups' data of the serum complement protein C3 and C4 were expressed by a value ($\mu\text{g/ml}$). For the data before the drug injections, an independent sample grouping t test method was used. Meanwhile, for the data before and after the drug injections, the data differences were obtained by matching the data t test method and the processed by SPSS 19.0 statistical software. The differences with a $P < 0.05$ were considered to have statistical significance.

Results

Serum complement protein C3 and C4 concentrations of the two groups before the intervention

Prior to the intervention, the serum complement protein C3 and C4 concentrations of the two mice groups were tested for normality using SPSS 19.0 software. The C3 concentration normality test results of the NS and BCG groups showed values of 0.99 and 0.357, respectively. The C4 concentration normality test results of the NS and BCG groups showed p values of 0.781 and 0.856, respectively. The data of the four groups fell within normal distributions (Table 1).

Two groups of independent sample t tests were carried out on the two groups of data, respectively. The two sets of mice C3 data showed the t test results of $t=0.339$ and $p=0.744$, respectively, while those of the C4 data showed t test results of $t=0.030$, and $p=0.976$, respectively. Prior to the intervention, there were no significant differences observed in the serum complement protein C3 and C4 concentrations between the two groups of mice ($p > 0.05$).

Serum complements protein C3 and C4 concentrations of the two mice groups after two weeks of intervention

In this study, after two weeks of the BCG and physiological saline injections, a tail scissor was used again to collect blood samples from all of the mice. A quantitative ELISA method was adopted to determine the serum complement protein C3 and C4 concentrations of the two mice groups after two weeks of intervention (Table 1).

The serum complement protein C3 and C4 concentrations after the intervention were subtracted from those found before the intervention, in order to obtain the complement protein C3 and C4

Table 1: Serum complement protein C3 and C4 concentrations of the two groups before and after two weeks of intervention (brackets).

| Control group | C3 (µg/ml) | C4 (µg/ml) | BCG group | C3 (µg/ml) | C4 (µg/ml) |
|---------------|------------------|--------------------|-----------|------------------|--------------------|
| NS1 | 68.51 (48.33) | 132.13 (115.55) | BCG1 | 24.82 (34.35) | 120.82 (136.43) |
| NS2 | 54.71 (44.53) | 141.68 (133.80) | BCG2 | 31.72 (23.78) | 111.27 (104.76) |
| NS3 | 22.53 (32.25) | 136.84 (141.86) | BCG3 | 74.25 (83.75) | 144.64 (160.04) |
| NS4 | 38.62 (24.16) | 112.35 (92.21) | BCG4 | 45.52 (40.33) | 137.78 (151.98) |
| NS5 | 48.96 (44.87) | 122.71 (131.32) | BCG5 | 37.47 (52.51) | 132.40 (153.25) |

concentration differences of paired mice before and after intervention. A *t* test method of the paired data was used for the *t* testing of the C3 and C4 concentration differences of the two mice groups before and after intervention, respectively. The results showed that the *t* test of the C3 concentration differences of the paired mice before and after intervention indicated a *t* value of 2.166, and a *p* value of 0.096. There were no significant differences observed in the C3 concentration differences between the two groups of mice before the intervention ($p > 0.05$). The *t* test of the C4 concentration differences of the paired mice before and after intervention indicated a *t* value of -2.800, and a *p* value of 0.049, and there were significant differences in the C4 concentration differences between the two groups of mice ($p < 0.05$), that meant that the C4 of the NS group decreased more significantly than that of the BCG group.

Renal pathological conditions

The renal arteriolar lesions of the BCG mice group were lighter than the saline group. They mainly manifested to a lesser infiltration degree of inflammatory cells, and less small vein thrombosis without obvious artery necrosis (Figure 1).

The renal interstitial edema of the BCG mice group was lighter than the saline group, similar to the infiltrations of the inflammatory cells around the renal tubules, interstitial necrosis degrees, and the degrees of renal tubular atrophy (Figure 2).

The glomerular lesions for lupus nephritis could be basically observed in the two groups' kidney biopsies. Then, statistics were carried out on the proportions of the various glomerular lesions, for example, from light to heavy (Table 2). The glomerular lesion severity was a check variable list, which was tested by a Kruskal Wallis-H (K) method, with a chi-square value of 10.8, and a *p* value of 0.001. It was considered that the proportion of the lighter mouse glomerular lesions of the BCG group was greater than that of the saline control group. The physiological saline group had larger proportions of glomeruli, with functions impaired or with no functions observed.

Survival situation

In regards to the survival dates of the two mice groups, the median survival periods of the mice in the saline and BCG groups were 48 and 60 days, respectively. A Kaplan Meier-method was used to analyze the survival situations of the two groups, and to prepare a K-M method curve (Figure 3). A logarithmic order (log-rank test) method was adopted to compare the survival curves of the two mice

groups, and the results were obtained as a chi-square of 3.900, and a *p* value of 0.048. It was considered that the survival curves of the two groups were different, and the survival rate of the BCG group was found to be higher than that of the saline group.

Discussion

Complement and hematologic system damages have been recognized as the criteria for the assessment of systemic lupus erythematosus (SLE) activities, and renal damages are the most common clinical manifestations of SLE [2]. In this study, due to the requirement of blood collections during the different experimental stages, the blood cell checks required more blood, which affected the survival periods of the mice, therefore, the changes in the hematologic systems were not taken as observation indexes. This study's research results showed that the BCG vaccine had the ability to improve the mouse serum complement C4 protein concentrations, reduce the kidney damages of experimental mice, and prolong the survival of the mice. These results suggested that the BCG vaccine may have therapeutic effects on systemic lupus erythematosus (SLE), which was basically consistent with the previous clinically observed results.

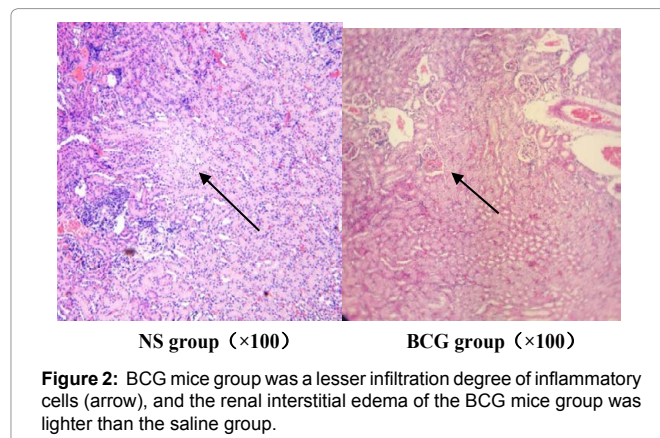
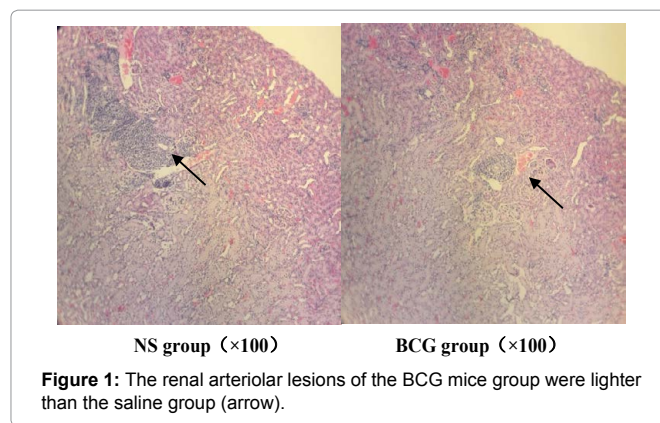


Table 2: The proportions of the various glomerular lesions between the BCG group and control group.

| Pathological classification | Minimal mesangial | Mesangial proliferative | focal LN | diffuse LN | Advanced sclerosing LN |
|-----------------------------|-------------------|-------------------------|----------|------------|------------------------|
| Control group | 3 | 18 | 93 | 28 | 8 |
| BCG group | 8 | 25 | 93 | 22 | 2 |

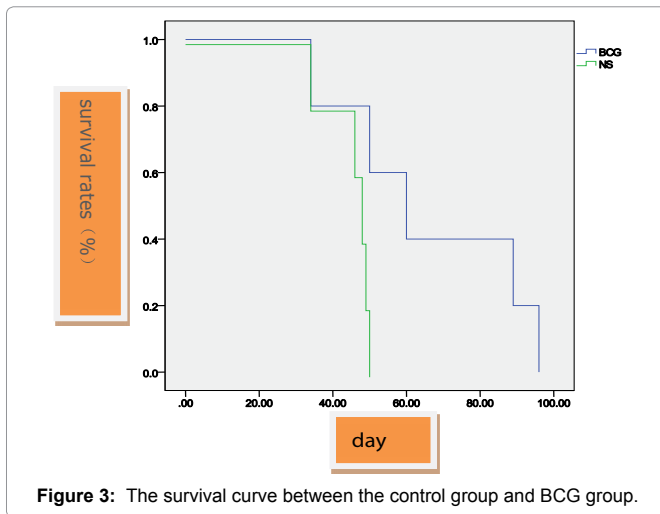


Figure 3: The survival curve between the control group and BCG group.

BCG is a live attenuated vaccine. It has been used to prevent tuberculosis infection for more than 80 years, and plays a major role in reducing the incidence of tuberculosis worldwide, especially in developing countries. During the last 10 years, many new roles have been found for BCG, such as promoting the production of interferon, as well as enhancing cellular immune functions [3,4,5], mononuclear cells activity, and phagocytosis of macrophage [6], and even is a strong antitumor [7]. According to the current data, it has not yet been applied in the treatment of SLE. However, there are a few reports available regarding the polysaccharide nucleic acid fraction of Bacillus Calmette Guerin (BCG PSN) and its cell immune regulation effects on SLE. Also, BCG-PSN has been determined to improve the level of the interferon in treatment groups, and has lowered SLE activities [8]. BCG-PSN is the polysaccharide nucleic acid fraction of Bacillus Calmette Guerin as an intravenous preparation, which has been approved for clinical use. However, due to weak immunogenicity, its therapeutic effects are active during use, and disappear when use is stopped. Therefore, there are rare similar research reports available. The BCG vaccine is injected by subcutaneous inoculation to uninterruptedly stimulate the immune cells, in order to produce continuous treatment effects without medication being daily required, which has great significance for future clinical applications.

According to the speculations of this study, the therapeutic role of the BCG vaccine in SLE treatment may be related to the regulation of the body's cellular immune system. The research results of Sun et al. [9] showed that the BCG had the ability to activate the TLR signaling pathways, and induce Th1-type cytokine secretions. Although SLE is a disease associated with humoral immunity, it has also now been confirmed to be related to cellular immunity, and is especially related to Th1/Th2 cytokine imbalances [10,11]. SLE belongs to the disease group caused by Th2-type cytokines dysfunction, similar to bronchial asthma. The use of corticosteroids and other immune inhibitors can treat and control SLE, as well as bronchial asthma activities. Also, glucocorticoid is a routine treatment of both these diseases. According to the Meta analysis recently published by El-Zein et al. [12], the BCG vaccine is a protective factor for asthma. Therefore, this study speculates that the BCG vaccination may also be a protective factor for SLE. Due to the difficulties in breeding experimental animal MRL/LPR mice, only a preliminary experiment was carried out in this study. When comparing the complement protein C3 changes in the mice, it was observed that the BCG group had a tendency to rise

compared to the control group. However, there were no statistical significances observed in the statistical check, which may have been associated with small size of the experimental sample. In the meantime, the next steps of the experimental process should be to enlarge the sample size, and to consider increasing the observational indices, such as urine protein, creatinine, and urea nitrogen.

In summary, it was found from preliminary experimental results of this study that the BCG vaccine was able to improve the mouse serum complement C4 protein concentrations, reduce the kidney damages of the experimental mice, and prolong the survival rate of the mice. These results suggested that the BCG vaccine may have therapeutic effects on systemic lupus erythematosus (SLE). These research results were encouraging, and laid a foundation for future research of the BCG vaccine's effect mechanism on SLE.

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