Effect of *Salvia miltiorrhiza* Bunge injection on anticardiolipin antibody production induced by β2 glycoprotein I

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**KEY WORDS** *Salvia miltiorrhiza* Bunge; glycoproteins; interleukin-2; T-lymphocytes

**ABSTRACT**

**AIM:** To explore the therapeutic effect and the mechanism of Chinese herbs on antiphospholipid syndrome (APS) by observing the effect of *Salvia miltiorrhiza* Bunge injection (SmBl) on anticardiolipin antibody (aCL) induced by β2 glycoprotein I (β2-GP I). **METHODS:** Sixty female mice randomly fell into 6 groups; group A, B, C, D was injected through abdominal cavity with different dosage of SmBl daily; after 14 d, group A, B, C, E was immunized with 150 μg of purified human β2-GP I in complete Freund's adjuvant subcutaneously; group F as control. The titre of aCL were detected by enzyme linked immunosorbent assay; subsets of T cell were grouped by streptavidin-biotin complex technique; and the activity of IL-2 was measured by MTT chromometry. **RESULTS:** (1) Compared with group E, the absorbance (A) of aCL in group A, B, and C decreased (P < 0.05 or P < 0.01). By linear correlation, the dosage is negatively correlated with the A values of aCL in 1, 2, and 3 weeks (P < 0.01). (2) Compared with group E, Th/Ts ratio was reduced in group A, B, and C (P < 0.05 or P < 0.01); there is no significant differences between group D and F (P > 0.05). By linear correlation, the dosage is negatively correlated with Th/Ts ratio (P < 0.01). (3) Compared with E, the activity of IL-2 in group B and C decreased significantly (P < 0.01). By linear correlation, there is negative correlation between dosage and IL-2 activity (P < 0.01). There is no significant difference between D and F (P > 0.05). (4) There is positive correlation between Th/Ts ratio and IL-2 activity.

**CONCLUSION:** The mechanism of suppressive effect of SmBl on aCL induced by β2-GP I may be realized by resuming the elevated Th/Ts ratio and IL-2 activity. The state that SmBl have no effect on normal mice indicates that SmBl has selective immunoregulative function.

**INTRODUCTION**

Antiphospholipid antibodies (aPL) are a family of autoantibodies. Their presence is associated with arterial/venous thrombosis and recurrent pregnancy loss. These clinical manifestations with the persistence of aPL are recognized as antiphospholipid syndrome (APS). aPL include lupus-type anticoagulant (LA), antiphospholipid antibody (aCL), and proteins (eg, prothrombin and β2 glycoprotein I). The ratio of APS related with aCL to LA is about 5:1-1. For APS, the common therapy include anticoagulation, anti-platelet, and thrombolysis2,3], but the effect of immunosuppressive agent is not confirmed4. Recently, investigation on therapeutic efficacy held by National Institute of Health (USA) indicated that there is still no ideal medicine regarding to APS. To provide evidence for the utilization of *Salvia miltiorrhiza* Bunge injection (SmBl) on APS, we observed the effect of SmBl on aCL induced by β2 glycoprotein I (β2-GP I).

**MATERIALS AND METHODS**

Isolation of human β2-GP I β2-GP I was isolated as described previously5,6,7 with some modifications. In brief, human normal mixed serum was precipitated with perchloric acid. This treatment was followed by ion-exchange chromatography on DEAE Fibrin and DEAE Sephadex A-50 (Pharmacia, Sweden). The sample was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Animal and grouping  Sixty female Kunming species of mice (provided by Xi-an Jiaotong University Experimental Animal Center, Grade II) weighing 20 g ± 2 g were randomly divided into 6 groups, 10 in each group. Group A, B, C, D was injected different dosage of SmBl (No 981203, Chiai Qinglei Bao Pharmaceutical Co, LTD, China) in order of 2, 5, 10, and 5 g·kg⁻¹·d⁻¹ ip daily, after 14 d, group A, B, C, and E was immunized sc with 150 μg of purified human β₂-GP 1 in complete Freund’s adjuvant once a week at multiple sites, three booster injections in all. Group F is control group.

Detection of aCL by enzyme linked immunosorbent assay (ELISA)  Getting blood once a week, four times in all, aCL was detected by ELISA as previously described 2. Absorbance readings were obtained using an enzyme labeled instrument (DG 3022A, Nanjing Eastern China Electron Tube Factory, China) at 450 nm.

Grouping of T cell subsets  To detect T cell subsets by strepavidin-biotin complex (SABC) with the use of a kit (Boshide Biotechnology Co, China); monocytes were separated by density gradient centrifugation, then dripped onto slide and fixed; pure methanol was diluted to 0.5 % by H₂O₂ and the slides were soaked in diluent methanol in 24 ℃; after antigen repairing and serum blocking, proper attendant rabbit-anti-mice McAb of L3T4 and Lyt-2 (Sigma Co., USA) then reagent SABC were dripped, after the slides were re-stained, dehydrated, cleared, and sealed, 200 cells were counted and the percentage of positive cell was calculated.

Measurement of IL-2 activity  The activity of IL-2 was measured by MTT chromometry 8. In brief, 100 μL proper attendant supernatant was added into 96-well flat-bottomed plates, and the IL-2 responsive cells, the spleen cells activated by ConA (Sigma Co., USA), were adjusted to 5 × 10⁶/L. After 48 h, the supernatant was removed, MITT solution was added in, and the mixture was incubated in 37 ℃ for 4 h; then acetylated isopropanol was dripped into the sediment, absorbance readings were obtained at 570 nm.

RESULTS

Variation in absorbance of aCL in different experimental groups  Compared with group E, the absorbance of aCL in group A, B, C was decreased (P < 0.05 or P < 0.01). By linear correlation, the dosage is negatively correlated with the absorbance in 1, 2, and 3 weeks (r = -0.981, -0.969, -0.963, respectively; P < 0.01) (Tab 1).

Alteration of T cell subsets  Compared with group E, T₉/T₅ ratio of group A, B, and C were decreased (P < 0.05 or P < 0.01). There is no significant differences between group D and F (P > 0.05). By linear correlation, the dosage is negatively correlated with T₉/T₅ ratio (r = -0.971, P < 0.01) (Tab 2).

Variation of activity of IL-2  The IL-2 activity of group A has no difference from group E (P > 0.05), but the IL-2 activity of group B and C decreased significantly (P < 0.01). By linear correlation, there is negative correlation between dosage and IL-2 activity (r = -0.955, -0.961, -0.973, respectively; P < 0.01). There is no significant difference between group D and F (P > 0.05) (Tab 3).

Relation between T₉/T₅ ratio and activity of

Tab 1. The absorbance value of aCL in mice ip preinjected with SmBl and sc immunized with purified human β₂-GP 1. n = 10. *P > 0.05, †P < 0.05, ‡P < 0.01 vs group E.

<table>
<thead>
<tr>
<th>Group</th>
<th>0 week</th>
<th>1 week</th>
<th>2 week</th>
<th>3 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.120 ± 0.012*</td>
<td>0.475 ± 0.018†</td>
<td>0.791 ± 0.018‡</td>
<td>0.971 ± 0.013‡</td>
</tr>
<tr>
<td>B</td>
<td>0.120 ± 0.012*</td>
<td>0.304 ± 0.017*</td>
<td>0.515 ± 0.020†</td>
<td>0.701 ± 0.095‡</td>
</tr>
<tr>
<td>C</td>
<td>0.121 ± 0.011†</td>
<td>0.199 ± 0.013‡</td>
<td>0.397 ± 0.014‡</td>
<td>0.604 ± 0.015‡</td>
</tr>
<tr>
<td>D</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E</td>
<td>0.120 ± 0.010</td>
<td>0.495 ± 0.021</td>
<td>0.820 ± 0.017</td>
<td>1.060 ± 0.023</td>
</tr>
<tr>
<td>F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

A: SmBl 2.5 g·kg⁻¹·d⁻¹ + β₂-GP 150 μg; B: SmBl 5 g·kg⁻¹·d⁻¹ + β₂-GP 150 μg; C: SmBl 10 g·kg⁻¹·d⁻¹ + β₂-GP 150 μg; D: SmBl 5 g·kg⁻¹·d⁻¹; E: β₂-GP 150 μg; F: blank control.
Tab 2. Alteration of T cell subset of mice ip preinjected with SmBI and sc immunized with purified human β2-GP 1. n = 10. ± s. *P > 0.05; **P > 0.05, ***P < 0.01 vs group E. ****P > 0.05 vs group F.

<table>
<thead>
<tr>
<th>Group</th>
<th>T4% cell/%</th>
<th>T2% cell/%</th>
<th>T4/T2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30.6 ± 2.7**</td>
<td>26.0 ± 1.6*</td>
<td>1.16 ± 0.04*</td>
</tr>
<tr>
<td>B</td>
<td>30.6 ± 2.4**</td>
<td>27.2 ± 1.3*</td>
<td>1.13 ± 0.08*</td>
</tr>
<tr>
<td>C</td>
<td>33.0 ± 2.3**</td>
<td>28.6 ± 1.3*</td>
<td>1.16 ± 0.04*</td>
</tr>
<tr>
<td>D</td>
<td>32.8 ± 2.3**</td>
<td>28.6 ± 1.6*</td>
<td>1.15 ± 0.12**</td>
</tr>
<tr>
<td>E</td>
<td>39 ± 3</td>
<td>25.8 ± 1.7*</td>
<td>1.51 ± 0.05</td>
</tr>
<tr>
<td>F</td>
<td>33.4 ± 2.5**</td>
<td>29.0 ± 1.7*</td>
<td>1.15 ± 0.08*</td>
</tr>
</tbody>
</table>

A: SmBI 2.5 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; B: SmBI 5 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; C: SmBI 10 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; D: SmBI 5 g.kg⁻¹.d⁻¹; E: β2-GP 1 150 µg; F: blank control.

Tab 3. Comparison of activity of IL-2 in mice ip preinjected with SmBI and sc immunized with purified human β2-GP 1. n = 10. ± s. *P > 0.05; **P < 0.01 vs group E. ***P > 0.05 vs group F.

<table>
<thead>
<tr>
<th>Group</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.85 ± 0.02%</td>
<td>0.79 ± 0.03%</td>
<td>0.72 ± 0.04%</td>
</tr>
<tr>
<td>B</td>
<td>0.71 ± 0.05%</td>
<td>0.62 ± 0.04%</td>
<td>0.58 ± 0.01%</td>
</tr>
<tr>
<td>C</td>
<td>0.58 ± 0.04%</td>
<td>0.51 ± 0.04%</td>
<td>0.45 ± 0.00%</td>
</tr>
<tr>
<td>D</td>
<td>0.52 ± 0.04%</td>
<td>0.43 ± 0.04%</td>
<td>0.37 ± 0.04%</td>
</tr>
<tr>
<td>E</td>
<td>0.62 ± 0.02%</td>
<td>0.81 ± 0.05%</td>
<td>0.74 ± 0.03%</td>
</tr>
<tr>
<td>F</td>
<td>0.53 ± 0.03%</td>
<td>0.46 ± 0.02%</td>
<td>0.38 ± 0.02%</td>
</tr>
</tbody>
</table>

A: SmBI 2.5 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; B: SmBI 5 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; C: SmBI 10 g.kg⁻¹.d⁻¹ + β2-GP 1 150 µg; D: SmBI 5 g.kg⁻¹.d⁻¹; E: β2-GP 1 150 µg; F: blank control.

IL-2 By linear correlation, there is positive correlation between T4/T2 ratio and activity of IL-2 in different dilutions (r = 0.977, 0.980, 0.972, P < 0.01).

DISCUSSION

Accompanied with thrombosis and recurrent abortion, aCL is of importance in clinical practice (9).

Wahl et al demonstrated that the risk for aCL associated thrombosis was higher, especially in patients with high titres. Although many hypotheses about the pathogenesis of thrombosis are based on experimental evidence, they are not generally accepted (11). The cause of aCL is unknown. It is presumed that a certain exotic peptide similar to β2-GP 1 is the genuine antigen. At present, heterogenous β2-GP 1 is the optimal antigen for model building, and immunization sc with purified β2-GP 1 can cause the production of aCL (12,13).

The optimal treatment on thrombosis induced by aPL is still debatable (14). The preferred therapy of most recrudescence symptom is anticoagulation. Combination of heparin and aspirin on women with recurrent fetal loss can enhance the survival rate from 40% to 80% (15), but the women are in a high risk of osteoporosis (16). Used to manage thrombo-embolism disease, warfarin may be the best effective medicine with international normalized ratio maintained at 3 (17), but warfarin can cause fetus deformity in pregnant woman. It is also controversial about whether prednisone should be used to treat APS. Though traditional Chinese medicine often get better effect on thrombosis caused by immune factor clinically, there is few reports about effective Chinese herb on APS.

Salvia miltiorrhiza Bunge, the commonly used herb, has many effects (10). The previous investigations about Salvia miltiorrhiza Bunge are frequently confined to hemostasis, rarely involved in immune aspect.

As shown in Tab 1, the absorbance of aCL were decreased in group A, B, and C (P < 0.05 or P < 0.01), which indicated that SmBI can perform suppressive effect on aCL; the dosage being negatively correlated with the A (P < 0.01) manifested that the suppressive effect is dosage-dependent. So, SmBI can block the process of thrombosis by restraining the production of aCL, which is the theoretical evidence for the application of SmBI on APS.

T cell is not only the effector cell of cellular immunity, but the important immune regulation cell. Among them, T1 cell and T2 cell exert a crucial role on regulation of cellular and humoral immunity; in the normal immune response, both interacts are in order to maintain the well-balanced immune function, which could not only eliminate antigenic foreign body, but be harmless to normal tissue. Compared with group E, T4/T2 ratio of group A, B, and C were decreased (P < 0.05 or P < 0.01), which suggested that SmBI can be helpful to APS by suppressing the elevated T4/T2 ratio.

Among the numerous immune regulation cytokines, IL-2 is in the center of the immune network, which is important in maintaining the natural function of immunity. IL-2 can accelerate the proliferation and differentiation of T cell and B cell, enhance the function of CTL and NK cell, keep the long-term growth of T cell in vitro, and promote the secretion of antibody by B.
Although the IL-2 activity in group A had no difference from group E ($P > 0.05$), the IL-2 activity in group B and C decreased significantly ($P < 0.01$), which indicated that SmIB can benefit autoimmune disease by affecting the activity of IL-2. Because there is positive correlation between $T_{H}/T_{S}$ ratio and activity of IL-2 in different dilutions ($P < 0.01$), and IL-2 is mainly produced by $T_{H}$, the imbalance of $T$ cell subsets may induce the alteration of IL-2 activity.

By linear correlation, the dosage of SmIB is negatively correlated with $T_{H}/T_{S}$ ratio and IL-2 activity ($P < 0.01$), which provide a reference for medication of SmIB.

As to $T_{H}/T_{S}$ ratio and IL-2 activity, there is no significant difference between group D and F ($P > 0.05$). Comparison with suppressive effect of SmIB above suggested that SmIB is selective in immune regulation, i.e., produce a marked effect on disordered, not on normal.

In conclusion, SmIB can not only produce anti-coagulation, anti-platelet effect, but regulate immunity. Consequently, SmIB takes on a broad prospect on the therapy of APS.

REFERENCES

位皮下注射 \( \beta_2 \)-GP I，E组为造模组；F组为空白对照组。用酶联免疫吸附实验法测定血清中的aCL；以链霉素亲和素-生物素复合物法检测外周血T细胞亚群；以四甲基偶氮唑蓝(MTT)比色法检测外周血白细胞介素-2(II-2)生物活性。结果：(1) 用药组血清aCL吸光度值较E组有不同程度降低(\( P < 0.05 \)或\( P < 0.01 \))；药物剂量与aCL吸光度值的变化成负相关(\( P < 0.01 \))。 (2) 与E组相比，A、B、C组均可降低\( T_H/T_S \)比值(\( P < 0.05 \)或\( P < 0.01 \))；D组与F组相比无统计学意义(\( P > 0.05 \))；药物剂量与\( T_H/T_S \)比值存在负相关(\( P < 0.01 \))。 (3) 与E组相比，A组对II-2活性无明显影响(\( P > 0.05 \))，而B、C组则使之明显降低(\( P < 0.01 \))；D组与F组无明显差异(\( P > 0.05 \))；药物剂量与II-2活性存在负相关(\( P < 0.01 \))。 (4) 经双变量直线相关分析，\( T_H/T_S \)比值和II-2生物活性呈正相关(\( P < 0.01 \))。 结论：丹参注射液可抑制 \( \beta_2 \)-GP I 诱导的aCL的产生，其作用机理可能是抑制\( T_H/T_S \)比值和II-2生物活性的升高；丹参注射液对正常小鼠无明显作用，提示其具有选择性免疫调节作用。