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Effect of *Salvia miltiorrhiza* Bunge injectio on anticardiolipin antibody production induced by β_2 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 injectio (*Sm*BI) on anticardiolipin antibody (aCL) induced by β_2 glycoprotein Ⅰ (β_2-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 *Sm*BI daily; after 14 d, group A, B, C, E was immunized with 150 μg of purified human β_2-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 chromatometry. **RESULTS**: (1) Compared with group E, the absorbance (A) of aCL in group A, B, and C was 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, T_H/T_S 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 T_H/T_S 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 T_H/T_S ratio and IL-2 activity in different dilutions ($P < 0.01$). **CONCLUSION**: The mechanism of suppressive effect of *Sm*BI on aCL induced by β_2-GP Ⅰ may be realized by resuming the elevated T_H/T_S ratio and IL-2 activity. The state that *Sm*BI have no effect on normal mice indicates that *Sm*BI has selective immunoregulative functive.

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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), anticardiolipin antibody (aCL), and proteins (eg, prothrombin and β_2 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 thrombolysis[2,3], but the effect of immunosuppressive agent is not confirmed[4]. 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 injectio (*Sm*BI) on APS, we observed the effect of *Sm*BI on aCL induced by β_2 glycoprotein Ⅰ (β_2-GP Ⅰ).

MATERIALS AND METHODS

Isolation of human β_2-GP Ⅰ β_2-GP Ⅰ was isolated as described previously[5-7] 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.

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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 *Sm*BI (№ 981203, Chiatai Qingchun Bao Pharmaceutical Co, LTD, China) in order of 2.5, 5, 10, and 5 $g \cdot kg^{-1} \cdot d^{-1}$ ip daily, after 14 d, group A, B, C, and E was immunized sc with 150 μg of purified human β_2-GP Ⅰ 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 were detected by ELISA as previously described[7]. Absorbance readings were obtained using a 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 streptavidin-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 H_2O_2 and the slides were soaked in diluent methanol in 24 ℃; after antigen repairing and serum blocking, proper attenuant 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 chromatometry[8]. In brief, 100 μL proper attenuant 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 5×10^6/L. After 48 h, the supernatant was removed, MTT solution was added in, and the mixture was incubated in 37 ℃ for 4 h; then acidulated isopropanol was dripped into the sediment, absorbance readings were obtained at 570 nm.

Statistics analysis The data were presented as $\bar{x} \pm s$. ANOVA and linear correlation analysis was performed by SPSS (version 8.0).

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, T_H/T_S 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 T_H/T_S 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 T_H/T_S ratio and activity of

Tab 1. The absorbance value of aCL in mice ip preinjected with *Sm*BI and sc immunized with purified human β_2-GP Ⅰ. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* group E.

Group	0 week	1 week	2 week	3 week
A	0.120 ± 0.012^a	0.475 ± 0.018^b	0.791 ± 0.018^c	0.971 ± 0.013^c
B	0.120 ± 0.012^a	0.304 ± 0.017^c	0.515 ± 0.020^c	0.701 ± 0.095^c
C	0.121 ± 0.011^a	0.199 ± 0.013^c	0.397 ± 0.014^c	0.604 ± 0.013^c
D	–	–	–	–
E	0.120 ± 0.010	0.495 ± 0.021	0.820 ± 0.017	1.060 ± 0.023
F	–	–	–	–

A: *Sm*BI 2.5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP Ⅰ 150 μg; B: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP Ⅰ 150 μg; C: *Sm*BI 10 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP Ⅰ 150 μg; D: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$; E: β_2-GP Ⅰ 150 μg; F: blank control.

Tab 2. Alteration of T cell subset of mice ip preinjected with SmBI and sc immunized with purified human β_2-GP I. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs group E. $^dP > 0.05$ vs group F.

Group	T_H cell/%	T_S cell/%	T_H/T_S ratio
A	38.6 ± 2.7^a	26.0 ± 1.6^a	1.48 ± 0.03^b
B	36.0 ± 2.4^b	27.2 ± 1.3^b	1.33 ± 0.03^c
C	33.0 ± 2.3^c	28.6 ± 1.3^c	1.16 ± 0.04^c
D	32.8 ± 2.3	28.6 ± 1.6	1.145 ± 0.028^d
E	39 ± 3	25.8 ± 1.7	1.52 ± 0.05
F	33.4 ± 2.5^c	29.0 ± 1.7^c	1.151 ± 0.028^c

A: *Sm*BI 2.5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; B: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; C: *Sm*BI 10 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; D: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$; E: β_2-GP I 150 μg; F: blank control.

Tab 3. Comparison of activity of IL-2 in mice ip preinjected with *Sm*BI and sc immunized with purified human β_2-GP I. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ vs group E. $^dP > 0.05$ vs group F.

Group	Dilution 1:2	1:4	1:8
A	0.854 ± 0.028^a	0.79 ± 0.03^a	0.72 ± 0.04^a
B	0.71 ± 0.05^c	0.62 ± 0.04^c	0.584 ± 0.019^c
C	0.58 ± 0.03^c	0.51 ± 0.04^c	0.454 ± 0.020^c
D	0.52 ± 0.04	0.45 ± 0.03	0.37 ± 0.04^d
E	0.872 ± 0.022	0.81 ± 0.05	0.74 ± 0.03
F	0.53 ± 0.03^c	0.464 ± 0.022^c	0.382 ± 0.024^c

A: *Sm*BI 2.5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; B: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; C: *Sm*BI 10 $g \cdot kg^{-1} \cdot d^{-1}$ + β_2-GP I 150 μg; D: *Sm*BI 5 $g \cdot kg^{-1} \cdot d^{-1}$; E: β_2-GP I 150 μg; F: blank control.

IL-2 By linear correlation, there is positive correlation between T_H/T_S 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[10] *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 β_2-GP I is the genuine antigen. At present, heterogenous β_2-GP I is the optimal antigen for model building, and immunization sc with purified β_2-GP I 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 recrudescent 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[18]. 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 *Sm*BI 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, *Sm*BI can block the process of thrombosis by restraining the production of aCL, which is the theoretical evidence for the application of *Sm*BI on APS.

T cell is not only the effector cell of cellular immunity, but the important immune regulation cell. Among them, T_H cell and T_S 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, T_H/T_S ratio of group A, B, and C were decreased ($P < 0.05$ or $P < 0.01$), which suggested that *Sm*BI can be helpful to APS by suppressing the elevated T_H/T_S 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

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cell. 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 *Sm*BI 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_{H1}, the imbalance of T cell subsets may induce the alteration of IL-2 activity.

By linear correlation, the dosage of *Sm*BI is negatively correlated with T_H/T_S ratio and IL-2 activity ($P < 0.01$), which provide a reference for medication of *Sm*BI.

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 *Sm*BI above suggested that *Sm*BI is selective in immune regulation, ie, produce a marked effect on disordered, not on normal.

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

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丹参注射液对 β_2 糖蛋白 I 诱导产生抗心磷脂抗体的影响

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关键词 丹参；糖蛋白类；白介素-2；T-淋巴细胞

目的：观察丹参注射液对 β_2 糖蛋白 I（β_2-GP I）诱导的抗心磷脂抗体（anticardiolipin antibody, aCL）产生的影响，以探讨其对抗磷脂综合征的治疗作用及可能的作用机制。**方法**：60 只雌性昆明种小鼠被随机分为六组，A、B、C、D 四组经腹腔注射不同剂量的丹参注射液；14 天后，A、B、C、E 四组多部

位皮下注射 β_2-GPⅠ. 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；以四甲基偶氮唑盐(MTT)比色法检测外周血白细胞介素-2 (IL-2)生物活性. **结果**：(1) 用药组血清aCL吸光度值均较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IL-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IL-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IL-2生物活性呈正相关($P<0.01$). **结论**：丹参注射液可抑制 β_2-GPⅠ诱导的aCL的产生，其作用机理可能是抑制 T_H/T_S 比值和IL-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.

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