Inhibitory effects of cryptoporus polysaccharide on airway constriction, eosinophil release, and chemotaxis in guinea pigs[1]

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ABSTRACT

AIM: To study effects of cryptoporus polysaccharide (CP) on antigen-induced bronchoconstriction, eosinophil peroxidase (EPO) release *in vivo*, and on platelet activating factor (PAF)-induced eosinophil chemotaxis *in vitro* in guinea pig. **METHODS:** The asthma model of guinea pig was formed with ovalbumin (OVA). The changes of lung resistance (R_L) and dynamic lung compliance (C_{dyn}), EPO level in bronchoalveolar lavage fluids (BALF) and eosinophil migration were determined. **RESULTS:** Pretreatment of CP at doses of 3, 9, and 27 mg/kg by intragastric gavage (ig), qd for 10 d, inhibited early asthma response in a dose-dependent manner. Inhibitory rates of mean increase value from 1 to 30 min of R_L were 34.8 %, 74.4 % (P<0.05), and 79.6 % (P<0.05), respectively. Inhibitory rate of mean reduction value of C_{dyn} were 22.9 %, 40.5 % (P<0.01), and 66.5 % (P<0.01), respectively. Pretreatment of CP at doses of 3, 9, and 27 mg/kg also inhibited late asthma response, and the reduction of EPO level in BALF were 3.1 %, 16.9 % (P<0.01), and 20.1 % (P<0.01), respectively. The inhibitory rates of CP at concentrations of 0.13, 1.3, 13, 130 nmol/L to eosinophil migration induced by PAF were 6.8 %, 17.2 % (P<0.05), 29.6 % (P<0.01), and 35.9 % (P<0.01). **CONCLUSION:** CP protects lung against increase of R_L and reduction of C_{dyn}, decreases EPO level in the asthma model, and inhibits eosinophil chemotaxis induced by PAF. The results suggest that CP may be a novel antiinflammatory agent for the treatment of asthma and allergic diseases.

INTRODUCTION

Cryptoporus volvatus grows wildly in China and its fruiting body has been used for asthma and bronchitis back to the fifteen century AD when the record of *Cryptoporus volvatus* appeared in "Materia Medica of Yunnan"[1]. Its natural resource is limited. Currently, we have obtained a great quantity of *Cryptoporus volvatus* through fermentation culture technology. This substance is the same as natural resource by identifying its physical and chemical properties[2]. Then, a low molecular weight acid polysaccharide with molecular weight of 15 000 daltons was isolated from *Cryptoporus volvatus*. Recently, Zhou JC found that cryptoporus volvatus (peck) schear inhibited the release of slow re-

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acting substance of anaphylaxis (SRS-A) from sensitized guinea pig lung and Schultz-Dale reaction of airway smooth muscle induced by antigen[3]. Cryptoporus polysaccharide (CP) can inhibit calcimycin-stimulated leukotriene D_4 (LTD_4) from normal guinea pigs lung *in vitro* and also inhibit eosinophil infiltration into lung tissue challenged by antigen in rats and in mice *in vivo* (unpublished data made by our Lab). In the present study, the effects of CP protecting against bronchoconstriction and EPO release from eosinophil in asthma model of guinea pig *in vivo*, and the effect of CP on eosinophil chemotaxis induced by PAF *in vitro* were evaluated.

MATERIALS AND METHODS

Materials and drugs Hartly guinea pigs of either sex, six month weighing 345 g±32 g were from Center of Medical School of Zhejiang University (Grade II, Certificate No 20010014 conferred by Zhejiang Medical Laboratorial Animal Administration Committee). Cryptoporus polysaccharide (TSBIO Science & Technology Co, Ltd, purity: 98 %, molecular weight: 15 000 daltons), Triton X-100 and Tris (Shanghai Sangon Biological Engineering Technology and Services Co, Ltd), Urethane and O-phenylenediamino dihydrochloride (OPD, Shanghai Chemical Reagent Company), Heparin sodium (Xuzhou Biochemical Pharmaceutical Factory), Sephadex G-200 (Pharmacia), RPMI 1640 MEDIUM (Hyclone), *L*-α-phosphatidylcholine, β-acetyl-γ-o-alkyl (platelet activating factor, PAF), ovalbumin (grade II), and PERCOLL (Sigma Chemical Company) were commercially available.

Sensitizing procedures The guinea pigs were sensitized im with 10 g/L ovalbumin 1 mL. The animals were used 24 d after aerosol antigen challenged.

Treatment procedures and the lung function measurement CP at doses of 3, 9, and 27 mg/kg qd (in our preliminary experiments, guinea pigs were administrated with CP at doses of 1, 3, 9, 27, and 54 mg/kg to determine dose-effect) were given ig on d 14 after sensitization for 10 d, salbutamol as positive control drug 4 mg/kg by ig 1 h before antigen challenge.

Guinea pig was anesthetized with urethane (1 g/kg, intraperitoneal, ip) at d 24. The trachea was cannulated and placed in a whole body plethysmography for measurement of lung resistance (R_L) and dynamic lung compliance (C_{dyn}). After 5 min for stabilizing preparation, increase of R_L and reduction of C_{dyn} of airway constriction were induced with exposure to an antigen inhalation of 10 g/L ovalbumin for 0.5 min generated by an ultrasonic nebulizer (particle size 1-5 mm; Model 402, Heli Medical Instrumental Factory, Shanghai) for the early asthma response. Increase value of R_L and reduction value of C_{dyn} were measured at the indicated time 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 min after the ovalbumin challenge[4].

EPO level measurement CP 3, 9, and 27 mg/kg qd were given ig for 10 d from d 14 after sensitization. Each guinea pig was challenged with 20 μL OVA intranasally qd for 5 d from d 6 of drug administration. Guinea pigs were killed on d 25. The lungs of the animals were washed three times with 5 mL of heparinized PBS. The total cells were counted in a hemocytometer and cells were resuspended in a concentration of 5×10^2 cells per μL. Substrate' pH (including 30 % H_2O_2 1.1 μL, Tris 1 mol/L 500 μL, Triton X-100 10 μL, OPD 0.9 g/L 100 μL, phosphate buffered solution 9388. 9 μL) was adjusted to 8 with HCl 1 mol/L. Five hundred cells per mL BALF 200 μL and substrate 100 μL were added in each well. The plate was then incubated for 0.5 h at 37.0 ºC in a 5 % CO_2 atmosphere. H_2SO_4 (4 mol/L) 30 μL were added to stop the reaction. Absorbence (A_{490}) of each well was determined[5].

Eosinophils migration Peritoneal eosinophils of guinea pigs were induced by sephadex G-200 (24 mg/kg, ip, on d 1, d 3). Peritoneal lavage was performed with 20 ml heparinized RPMI1640 on d 5. Heavy solution was made by 9 parts of Percoll mixed with 1 part of 10×concentrated phosphate-buffered D-Hanks solution. Separation solution was 6.5 parts of heavy solution mixed with 3.5 parts of D-Hanks solution. Separation solution 1.5 mL was laid in the tube and heparinized peritoneal lavage was layered on it. The tubes were then centrifuged in a horizontal rotor at 500×*g* for 20 min at room temperature. The eosinophils layer was removed into another tube and 3 mL RPMI 1640 was added, then the tubes were centrifuged in an angle rotor at 50×*g* for 10 min at 22 ºC-25 ºC temperature[6]. The supernatant fluids were abandoned. Cell purity and viability were determined by using Wright-Giemsa stain and the Trypan blue dye exclusion test, respectively. Eosinophils with a purity and viability of more than 95 % were used for experiments. The concentration of eosinophils was adjusted to 1×10^3 cells per mL. Chemotaxis assays were performed in a 48-well microchemotaxis chamber. The bottom wells of the chamber were filled with 29 μL chemoattractant agent

(PAF 0.4 nmol/L–40 mmol/L, respectively), whereas the upper wells were filled with eosinophils 30 μL and RPMI 1640 15 μL. The bottom and upper layers were separate by a polycarbonate filter of 5 μm (Nucleopore). The chamber was then incubated for 2 h at 37.0 ºC in a 5 % CO_2 atmosphere. At the end of the incubation period, the filter was removed, washed, stained with hematoxyllin for 1 min, and cells were counted on a glass slide. Experiments were replicated three times. Chemotaxis was determined by counting eosinophils that had migrated completely through the filter in three high-power fields (HPF: 200×) per well randomly[7]. In the following experiment the bottom wells were filled with PAF at the concentration of 400 nmol/L because it caused the maximum chemotaxis effect, and the upper wells were filled with eosinophils 30 μL that had been treated with 15 μL CP at concentrations of 0.13, 1.3, 13, and 130 nmol/L, respectively. The following procedures were same as the former chemotaxis assays.

Statistical analysis Data were expressed as Mean±SD, and analyzed by Dunnett's test.

RESULTS

Effect of CP on airway constriction in antigen challenged guinea pigs There was no significant difference in basal R_L and C_{dyn} between each group. Inhaled antigen caused bronchoconstriction that peaked within 60 s. Pretreatment with CP at dose of 3, 9, and 27 mg/kg (ig) inhibited early asthma response of sensitized guinea pigs induced by aerosol OVA in a dose-dependent manner (Tab 1, 2). Regarding R_L of control group as 100 %, inhibitory rate of mean increase value from 1 to 30 min of R_L was 34.8 %, 74.4 % (P<0.05), and 79.6 % (P<0.05), respectively. Regarding C_{dyn} of control group as 100 %, inhibitory rate of mean reduction value of C_{dyn} was 22.9 %, 40.5 % (P<0.01), and 66.5 % (P<0.01), respectively. Salbutamol 4 mg/kg (ig), as a positive control drug, the inhibitory rate of mean increase value from 1 to 30 min of R_L and C_{dyn} was 66.8 % (P<0.05), and 64.1 % (P<0.01), respectively.

Effect of CP on EPO level in antigen challenged guinea pigs Pretreatment with CP at dose of 3, 9, and

Tab 1. Inhibition of cryptoporus polysaccharide (CP) ig on ovalbumin induced increase of lung resistance (R_L) of guinea pigs. Control: n=6; CP 3 mg/kg: n=9; CP 9 mg/kg: n=6; CP 27 mg/kg: n=7; salbutamol 4 mg/kg: n=8. Mean±SD. [b]P<0.05 *vs* control.

		Lung resistance (R_L)/%										
Group	n	0 min	1 min	2 min	3 min	4 min	5 min	10 min	15 min	20 min	25 min	30 min
Control	6	100	165±18	254±60	241±47	224±54	219±59	158±16	143±14	141±12	109±8	104±11
CP 3 mg/kg	9	100	151±22	203±24	176±22	167±17	156±14	140±20	128±14	124±14	132±17	118±13
CP 9 mg/kg	6	100	120±10	139±18	124±9[b]	126±5	122±3	135±13	107±13	110±10	107±5	105±4
CP 27 mg/kg	7	100	117±6[b]	122±6[b]	118±7[b]	121±5	124±6	113±9[b]	117±7	111±8	106±6	107±4
Salbutamol 4 mg/kg	8	100	125±11	126±11[b]	126±11[b]	127±11[b]	131±16[b]	129±18	119±14	116±14	131±17	122±20

Tab 2. Inhibition of cryptoporus polysaccharide (CP) ig on ovalbumin induced reduction of dynamic lung compliance (C_{dyn}) of guinea pigs. Control: n=6; CP 3 mg/kg: n=9; CP 9 mg/kg: n=6; CP 27 mg/kg: n=7; salbutamol 4 mg/kg: n=8. Mean±SD. [b]P<0.05, [c]P<0.01 *vs* control.

		Dynamic lung compliance (C_{dyn})/%										
Group	n	0 min	1 min	2 min	3 min	4 min	5 min	10 min	15 min	20 min	25 min	30 min
Control	6	100	65±18	49±15	48±9	48±3	52±1	72±11	78±12	79±10	96±9	100±10
CP 3 mg/kg	9	100	67±7	56±7	67±8	67±8	71±8	80±8	82±9	88±9	85±8	96±7
CP 9 mg/kg	6	100	73±9	67±10	70±9	77±9[b]	82±7[c]	78±9	92±13	87±14	94±8	95±5
CP 27 mg/kg	7	100	81±6	76±8	79±8[b]	79±7[c]	80±5[c]	94±6	97±6	102±6	103±7	105±6
Salbutamol 4 mg/kg	8	100	83±8	82±8[b]	89±9[b]	91±10[c]	86±9[b]	90±10	86±10	93±10	93±13	95±12

27 mg/kg (ig) inhibited EPO level in BALF of antigen sensitized and challenged guinea pigs by 3.1 %, 16.9 % (P<0.01), and 20.1 % (P<0.01), respectively (Tab 3). As a positive control, ketotifen 5 mg/kg inhibited EPO release by 19.0 % induced by antigen (P<0.01).

Tab 3. Inhibition of cryptoporus polysaccharide (CP) ig on eosinophil peroxidase level in bronchoalveolar lavage fluids of guinea pigs. Mean±SD. ^cP<0.01 *vs* control.

Group (mg/kg)		Number of animal	Number of sample	$A_{490\ nm}$
Control		3	15	0.52±0.06
CP	3	3	13	0.50±0.03
	9	4	16	0.43±0.04^c
	27	3	14	0.41±0.03^c
Ketotifen	5	3	14	0.42±0.07^c

Tab 4. Eosinophils chemotaxis induced by platelet activating factor. n=24 samples. Mean±SD. ^cP<0.01 *vs* RPMI1640.

		Eosinophils number in 1/20 HPF
RPMI 1640		11.29±2.26
PAF /nmol·L^{-1}	0.4	25.08±6.89^c
	4	29.46±7.41^c
	40	33.42±16.55^c
	400	48.81±16.95^c
	4000	33.28±17.23^c
	40000	20.28±13.99^c

Effect of CP on eosinophils chemotaxis PAF 0.4 nmol/L-40 mmol/L caused a bell-reaction curve of eosinophils migration and the number of chemotaxis eosinophils were increased by 1.2, 1.6, 2.0, 3.3, 1.9, and 0.8 times, respectively (Tab 4). CP 0.13, 1.3, 13, 130 nmol/L inhibited PAF (400 nmol/L)-induced eosinophils chemotaxis in a concentration-dependent manner (Tab 5). Inhibitory rate of CP were 6.8 %, 17.2 % (P<0.05), 29.6 % (P<0.01), 35.9 % (P<0.01), respectively.

Tab 5. Inhibition of cryptoporus polysaccharide on eosinophils chemotaxis. n=36 samples. Mean±SD. ^bP<0.05, ^cP< 0.01 *vs* RPMI1640.

		Eosinophils number in 1/20 HPF
RPMI 1640		37.33±13.50
CP /nmol·L^{-1}	0.13	34.79±8.17
	1.3	30.92±4.82^b
	13	26.29±7.03^c
	130	23.92±7.90^c

DISCUSSION

In this study, we first demonstrated that CP inhibited antigen inhalation challenge-induced airway constriction in sensitized guinea pigs. CP had no direct relaxation effect on airway smooth muscle *in vitro*, but it inhibited Schultz-Dale constriction reaction of airway smooth muscle induced by antigen and leukotriene D_4 (LTD_4) from normal guinea pigs lung stimulated by calcimycin *in vitro*[3]. Recently, the stabilizing effect of CP on mast cell membrane and regulating effect of CP on Th1/Th2 cells balance have been found (unpublished data made by our Lab). Together, these evidences suggest that CP had effects on histamine, leukotrienes, and cytokines produced from mast cells in asthmatic airways.

Eosinophils are the predominant inflammatory cell type found in the airway of asthmatics and these cells have been suggested to play a role in the pathogenesis of asthma. Activated eosinophil are capable of releasing a variety of inflammatory mediators including cationic proteins such as EPO, eosinophil derived neurotoxin and eosinophil cationic protein. The levels of these proteins are increased 24 h after bronchial allergen challenge and there is evidence to suggest that these cationic proteins damage the respiratory epithelium and may contribute to the development of bronchial hyperresponsiveness. Furthermore an increase in the number of activated eosinophils present in the airway has been demonstrated to correlate well with disease severity and a large number of eosinophils and their granule products have been found in and around the asthmatic airway[8]. In this experiment we choose OPD as the substrate to determine EPO level in BALF[9]. Consistent with other studies[10], EPO activity in BALF was increased in sensitized guinea pigs after antigen challenge in our experiments. CP decreased the release of EPO and protected airway and lung tissues.

Chemotaxis is the directed cell migration toward a

chemical stimulus. By this crucial mechanism eosinophils migrate into inflammatory sites. *In vitro*, a number of stimuli have been identified that are potent and effective eosinophil chemoattractants. The most notable of these are PAF[11] and some members of chemokine family such as eotaxin, RANTES[12,13]. PAF activates tyrosine kinase and PI_3 kinase in eosinophils, then activates mitogen-activated protein kinases to increase intracellular Ca^{2+} which is related to eosinophils' movement[14]. CP significantly attenuated PAF-induced chemotaxis in a concentration-dependent manner. CP has an inhibitory effect on asthmatic airway inflammation by acting on eosinophil chemotaxis.

In conclusion, CP markedly inhibited airway constriction and EPO release from eosinophil in asthmatic model of guinea pig and significantly inhibited eosinophil chemotaxis. The results suggest that CP may be a novel antiinflammatory agent for the treatment of asthma and allergic diseases.

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