นิพนธ์ต้นฉบับ

ซัลเฟตไคโตซานยับยั้งการสร้างอิมมูโนโกลบูลิน ของเม็ดเลือดขาวชนิดเซลล์เดี่ยว

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บทคัดย่อ

ไคโตซานโปลีซัลเฟตซึ่งสังเคราะห์ได้เองในห้องปฏิบัติจำนวน 3 ตัวอย่างที่มีขนาดน้ำหนักโมเลกุลเฉลี่ยต่างกัน ได้รับการตรวจสอบคุณสมบัติทางชีวภาพแล้วว่ามีฤทธิ์แรงในการยับยั้งการแข็งตัวของเลือด ถูกนำมาศึกษาความเกี่ยวข้องต่อการสร้างและหลั่งอิมมูโนโกลบูลินชนิดต่างๆ ในเม็ดเลือดขาวชนิดเซลล์เดี่ยวของคนเมื่อได้รับการกระตุ้นด้วยโพควีดไมโตเจน การทดสอบใช้เม็ดเลือดขาวชนิดเซลล์เดี่ยวความเข้มข้น $5x10^5$cells/well เพาะเลี้ยงเซลล์ในภาวะที่มีและไม่มีสารทดสอบในความเข้มข้นต่างๆ (0-8 มค.ก/มล.) และ / หรือโพควีดไมโตเจน (2.5 มค.ก /มล.) การศึกษาทำเปรียบเทียบกับเพนโตซานโปลีซัลเฟตและสารมาตรฐานเฮพาริน พบว่าไคโตซานโปลีซัลเฟตและเพนโตซานโปลีซัลเฟตสามารถยับยั้งการสร้างและหลั่งอิมมูโนโกลบูลินได้อย่างมีนัยสำคัญ ($P<0.01$) และการยับยั้งซึ่งมีลักษณะสอดคล้องกับความเข้มข้นนี้ไม่ได้เป็นผลจากพิษของสารทดสอบต่อเซลล์ เนื่องจากการทดสอบความมีชีวิตของเซลล์มีค่ามากกว่า 90% เฮพารินสามารถยับยั้งได้เช่นกัน แต่ผลไม่ชัดเจนอย่างมีนัยสำคัญ กล่าวได้ว่าไคโตซานโปลีซัลเฟตซึ่งมีฤทธิ์ยับยั้งการแข็งตัวของเลือดนี้สามารถยับยั้งการแบ่งตัว การสร้างและหลั่งอิมมูโนโกลบูลินของเม็ดเลือดขาวชนิดเซลล์เดี่ยวได้ และความเกี่ยวข้องนี้น่าจะมีปัจจัยจากจำนวนหมู่ซัลเฟตและรูปแบบการเติมหมู่ซัลเฟต และ / หรือขนาดน้ำหนักโมเลกุล อย่างไรก็ดีนับเป็นรายงานแรกที่ศึกษาผลกระทบของไคโตซานโปลีซัลเฟตที่มีต่อการทำงานของเซลล์ในระบบภูมิคุ้มกันชนิดสารน้ำของร่างกายคน วารสารเทคนิคการแพทย์เชียงใหม่ 2546; 36: 120-128.

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Abstract: Sulfated Chitosan Inhibits Immunoglobulin Production of Human Peripheral Blood Mononuclear Cells

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The 3 isolated fractions of chitosan polysulfate (CPS) with different average molecular weight (P1, P2 and P3) with high potency in anticoagulant activity was synthesized in our laboratory. Its involvement in immunoglobulin production using pokeweed mitogen (PWM) as the stimulant was elucidated. The test condition was performed using PBMCs ($5x10^5$cells/well) in the presence or absence of PWM (2.5 μg/mL) and various concentrations (0-8 μg/mL) of CPS in comparison to pentosan polysulfate (PPS) and heparin. The results showed that CPS and PPS could significantly inhibited PWM stimulated immunoglobulin production of PBMCs ($P<0.01$). The inhibition effect was in dose dependent manner. This effect was not significantly observed in case of heparin. The decreasing in proliferation was not due to the toxicity of products as the dye exclusion assay showed > 90% cell viability as was observed in cell cultured in the presence of PWM alone. Our results suggested that CPS could inhibit PWM induced immunoglobulin production of human PBMCs. This might be affected from both specific sequence of sulfation patterns and size of polymer chains. In addition, this is the first report of immunologic effect of CPS on human PBMCs. **Bull Chiang Mai Assoc Med Sci 2003; 36: 120-128.**

Key words: chitosan polysulfate, immunoglobulin production, PBMCs, heparin, pentosan polysulfate, immunologic effect.

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1. Introduction

Chitosan, a natural cationic polymer, is obtained from N-deaceylation of chitin. Chitin, the second most abundant natural polymer, is harvested mainly from the exoskeleton of marine crustaceans such as crabs and shrimps.[1] Chitosan has been proposed for a number of medical and pharmaceutical applications.[2] It is a simple β–(1→4) glycans composed of 2-acetamido-2-deoxy-D-glucopy-

ranose or 2-amino-2-deoxy-D-glucopyranose residues, and it has served as key starting materials for the efficient preparation of bioactive polysaccharides having anticoagulant activity,[3-5] immunomodulating effects,[6] and inhibitory effects on lung metastasis of melanoma cells.[7, 8] We have modified chitosan derived from marine crab shell using chlorosulfonic acid in a mixture of dimethylformamide (DMF) as a sulfate donor by a semi-heterogeneous conditions mentioned elsewhere.[9,10] The sulfated chitosan obtained was demonstrated to have a high potent in anticoagulant activities in the mechanism that closed to the standard therapeutic heparin and the well-known synthetic sulfated polysaccharide, pentosan polysulfate which were reported to cause thrombocytopenia dues to the induction of antiplatelet antibodies.[11] Therefore, our product might represent a new therapeutic drug in the future. There were many reports in sulfate modification of chitin/ chitosan and its derivatives and demonstrated its anticoagulant activities, however, there was no reports about its involvement in the human immune response. It was of interest that chitosan polysulfate with anticoagulant activity may involve in the immunoglobulin production of human peripheral blood mononuclear cells.

To address whether our products show any involvement in the human immunologic response if it will be used as a medical compound, effect on immunoglobulin production of human PBMCs was studied. The purpose of this study was to clarify the effect of synthetic chitosan polysulfate in comparison with those heparin and pentosan polysulfate on the pokeweed mitogen (PWM) stimulated PBMC proliferation. We reported the significant inhibition effect of sulfated chitosan and pentosan polysulfate on immunoglobulin production induced by PWM. The effect was in dose dependent manner and this effect was not observed in case of heparin.

2. Materials and methods

Sulfated chitosan, - 2-deoxy-2-sulfoamido-3,6-di-*O*-sulfo-(1→4)-b-D-glucopyranan (**2**). Chitosan, (**1**) 2-amino-2-deoxy-(1→4)-b-D-glucopyranan was sulfate substituted by the method mentioned elsewhere.[10] Sulfated chitosan (**2**) was applied to a column (1.6x100 cm) of Sepharose CL-6B equilibrated in phosphate-bufferred saline, pH 7.2 (PBS). The column was eluted with the same buffer at flow rate of 20 ml/hr and the fractions of 2 ml were collected and investigated by the 1,9-Dimethylmethylene Blue (DMBA) binding assay. All fractions showing positive DMBA were separated into three major peaks (P1-P3) at K_d 0.16 and 0.58. Following dialysis against distilled water and lyophilyzation, each peak was purified on a Mono Q column-FPLC (HR 5/5) (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris/ HCl, pH 8.0. The column was eluted with a linear gradient of 0-2.0M NaCl and the fractions of 1ml were collected and assayed

by DBMA. Finally, all DMBA positive fractions were pooled, dialyzed against distilled water and lyophilized. The yield of (2) was 55%, $[\alpha]_D$ -14.3 (c 0.5, water); IR (KBr)ν_{max} :3500-3200 (OH, NH), 1640, 1660 (C=O), 1240 (S=O), and 800 (C-O-S) Anal. Calcd. For $[C_6H_8O_4(C_2H_3O)_{0.12}(SO_3Na)_{2.23}(H)_{0.65}.3.7H_2O]_n$: C, 15.83; H, 3.34; N, 3.08; S, 16.18; Na, 11.63. Found: C, 16.99; H, 3.07; N 3.12; S, 15.64; Na, 11.33. ^{1}H NMR (DSS, D_2O): δ 4.92 (br d, 1H, H-1), 4.56 (br t, 1H, H-3S), 4.25 (m, 2H, H-4, H-5), 3.97 (br d, 2H, H-6S), 3.69 (br t, 1H, H-6), 3.45 (br t, 1H, H-2S), 3.15 (br t, 1H, H-2), 2.05 (s, 3H, CH_3).

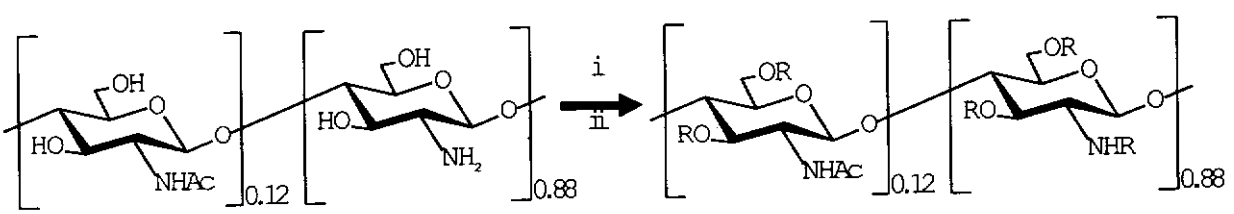
General methods.- 1H NMR spectra were recorded on a Jeol JNM-A500 instrument with DSS as the internal reference. IR spectra were recorded on a Jasco IR-810 instrument as KBr discs. Optical rotations recorded on a Perkin-Elmer 343 digital polarimeter at 28 °C. The average molecular weight of 1 and 2 were determined viscosmetrically using an Oswald type viscometer and applying the Mark-Houwink equation $[\eta] = 7.8x10^{-3}Mv^{0.76}$ (0.3M AcOH, 0.2M NaOAc, 25 °C)[15] and [h] = $1.75x10^{-5}Mv^{0.98}$ (0.1M NaCl,25 °C),[3] respectively. CHNS analysis were performed on a PE2400 Series 2 Perkin-Elmer instrument.

Assays for the effect on immunoglobulin production.- Human peripheral blood mononuclear cells (PBMCs) were isolated from three healthy volunteers by Ficoll-Hypaque gradient centrifugation technique using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Cells were washed 4 times with RPMI-1640 to eliminate plasma immunoglobulins and resuspended in PMI-1640 supplemented with 10% fetal calf serum. Cells were cultured at a final concentration of 5 x 10^5 cells/ml in the presence or absence of P1-P3, PPS and heparin (0-8 μg/ml). Cell cultures were incubated for 4 hrs in a CO_2 incubator at 37 °C, 95% humidity before adding of PWM (Sigma-Aldrich, St. Louis, MO) (2.5 μg/ml). The cultures were incubated for another 10 days. Cell culture supernatant was collected and centrifuged at 500g for 10 minutes. Goat anti human IgA, -IgM, -IgD, -IgE, and –Ig polyvalent (Igs) and rabbit anti human IgG (Sigma-Aldrich, St. Louis, MO) were diluted 1:1000 in 0.01M carbonate/bicarbonate buffer pH 9.6 and coated 100 μl/well on microtiter plates at 4 °C overnight. Plates were washed 3 times with 0.05%Tween-20 in PBS pH 7.2 (PBST) before blocking with 200 μl/well of 5% BSA/PBST for 1 hr at 37 °C. Plates were washed 3 times with PBST and 1:10 diluted cell cultured supernatants were added 100 μl/well. Plates were then incubated for another 2 hrs at 37 °C and washed 3 times. Peroxidase conjugated goat anti human Igs (1:2500) was added 100 μl/well into the set of goat anti human Igs coated as a primary antibody. Peroxidase rabbit anti human Igs (1:20,000) was added 100 ml/well into the set of rabbit anti human IgG coated as a primary antibody. The reaction was performed for 2 hrs at 37 °C and washed 4 times before adding of peroxidase substrate, TMB (Sigma-

Aldrich, St. Louis, MO) 200 µl/well. Plates were incubated 30 minutes at room temperature and the reaction was stopped by adding of 2.5N H_2SO_4 50 µl/well. Plates were read at 405 nm.

3. Results and discussion

Chitosan from marine crab shells with degree of deacetylation of 0.88[1] was sulfate substituted by the method of Gamzazade[9] with some modifications in a semi-heterogeneous conditions (Scheme 1). The 2-deoxy-2-sulfoamido-3,6-di-O-sulfo-(1→4)-β-D-glucopyranan[2] was obtained in 55% yield after purification by Mono Q ion-exchange FPLC. It was the sodium salt, as determined by atomic absorption spectrometry. Characteristic absorptions in the IR spectrum at 800 and 1240 cm^{-1}, due to sulfo groups, were assigned to C-O-S and S=O bond stretching, respectively and the degree of substitution was 2.23. The ^1H NMR analysis showed the complete substitution. The observed chemical shifts were d 3.97, 4.56, and 3.45 ppm, for H-6S, H-3S, and H-2S, respectively (Fig.1). The major peak at 3.45 and a minor one at 3.15 indicated the substitution of H-2S and also confirmed the decrease of free amino groups in (**2**), as compared to the starting chitosan. This decrease was confirmed by ninhydrin assay.[12] (**2**) was separated into three fractions with different average molecular weights (Mv) of 6.8, 3.5, and 2.0 x 10^4 using Sepharose CL-6B column. The anticoagulant activity of each fraction was studied in comparison to pentosan polysulfate (PPS) and therapeutic heparin. The results showed that our products has two main effects on the factors in the coagulant pathway, inhibition of FXa activity through the interaction with antithrombin III, and direct inhibition of thrombin activity.



1 2

Scheme 1 1; chitosan, 2; sulfated chitosan, i; chlorosulfonic acid/DMF, ii; at room temperature for 5 hrs, R = SO_3Na or H^+

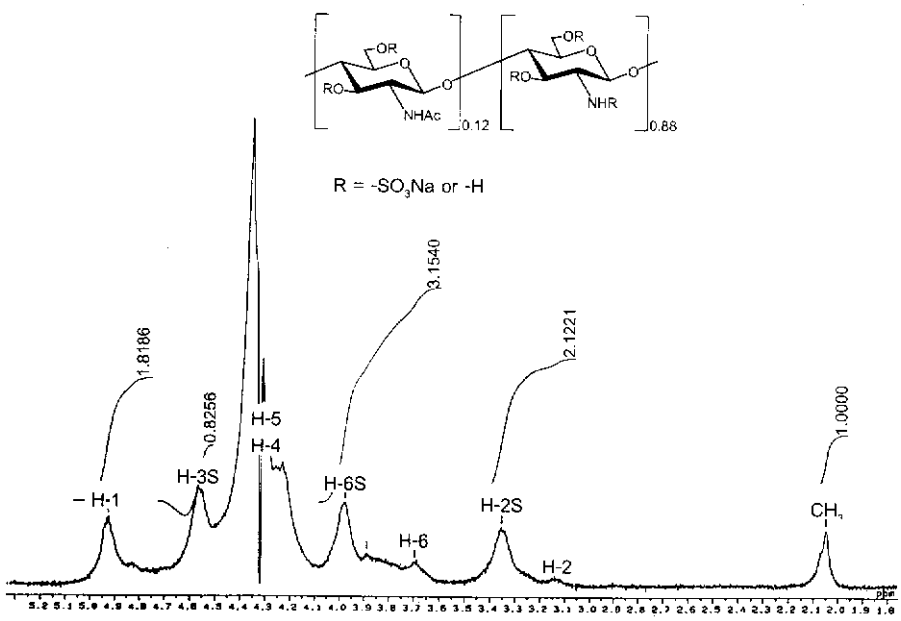


Figure 1 ^{1}H-NMR of CPS (D_2O). H-3S, H-6S and H-2S; sulfate positions

Chitosan was once reported to increase IgM production in human-human hybridoma cell lines and PBMCs.[13] It induced only IgM and has no effect on other subtype. However, there was no report of CPS and its effect on the immunoglobulin production on normal human PBMCs. To address this effect, an assay was performed using PWM as a stimulant. The effect of tested materials on the immunoglobulin production of PBMCs in the absence of PWM was also performed in parallel. Each donor demonstrated the individual characteristics in the immunoglobulin production which depended on individual basic status. The results demonstrated that PBMCs from different donors had distinct ability to produce immunoglobulin both in terms of levels and classes (Fig 2). In the absence of PWM, tested materials did not affect the immunoglobulin production even at the high concentrations of 8 mg/ml. The inhibitory effects on immunoglobulin production stimulated by sub-optimal dose of PWM were observed in all tested CPS (P1-P3). In addition, there was no correlation between size and bioactivity. More interesting, the CPS could significantly inhibited IgM production compared to other immunoglobulin classes ($p<0.01$). In contrast, PPS showed the most effective inhibitory effect among all tested materials while heparin demonstrated the specific different inhibitory patterns. The decreasing in proliferation was not due to the toxicity of products as the dye exclusion assay showed > 90% cell viability as was observed in cell cultured in the presence of PWM alone (data not shown). The inhibitory effect of heparin was

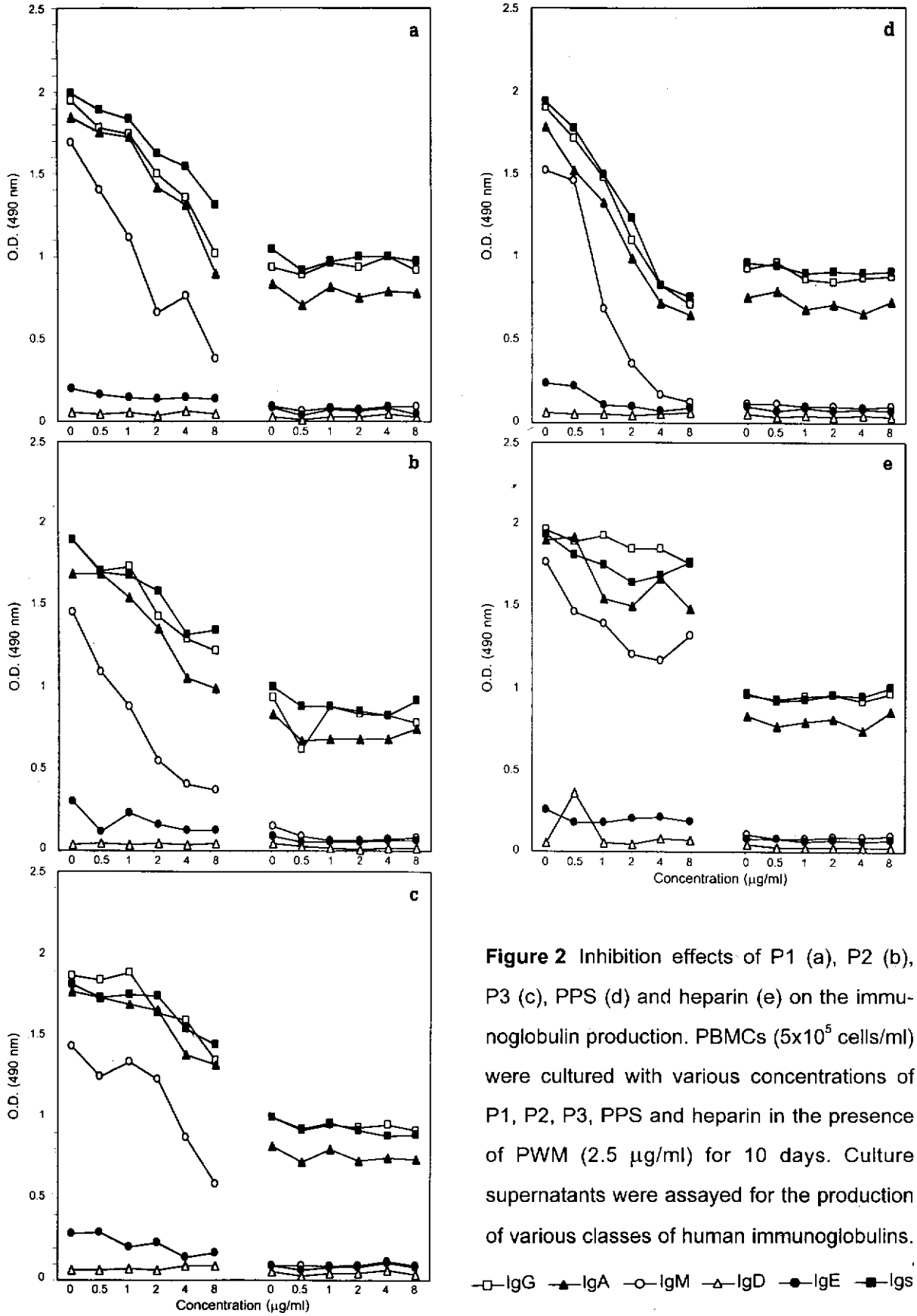


Figure 2 Inhibition effects of P1 (a), P2 (b), P3 (c), PPS (d) and heparin (e) on the immunoglobulin production. PBMCs ($5x10^5$ cells/ml) were cultured with various concentrations of P1, P2, P3, PPS and heparin in the presence of PWM (2.5 μg/ml) for 10 days. Culture supernatants were assayed for the production of various classes of human immunoglobulins.

—□—IgG —▲—IgA —○—IgM —△—IgD —●—IgE —■—Igs

not as strong as CPS and PPS and was not in dose dependent manner. PWM is a mitogen that has a specific binding property to N-acetylglucosamine and it can stimulate both T and B cell. Stimulation of B cell is independent of T cell help. First proposed mechanism of inhibitory effect was that it could be blocked by binding to its specific sites on tested materials. This effect, therefore, reduced the concentration of PWM in the culture. However, this proposed mechanism was not conclusion. The reason were that, firstly, the degree of acetylation in CPS is only 0.12 and moreover it was demonstrated that degree of sulfation was 2.23 which revealed that almost all of N-acetylglucosamine units in the backbone were substituted. Therefore, the binding sites of PWM in the CPS is very rigid. Secondly, the sub-optimal concentration of PWM was used in the assay. The sub-optimal concentration was optimized in order to differentiate the effect of tested materials on the assay. Therefore, the inhibitory effect could not be caused by the low concentration of mitogen used. Thirdly, PPS which is the synthetic sulfated polysaccharide has its own natural backbone of xylan and which has no specific binding sites for PWM could strongly inhibit the effect of PWM. The second proposed mechanism was that the binding of PWM and its specific oligosaccharides on the membrane glycoprotein of T lymphocytes may be interfered with highly anionic charge of tested materials. In addition, the interaction between polysaccharide sulfate and may be its specific receptor on the cell membrane.[14] The mechanism of CPS and PPS in inhibiting the PWM stimulated immunoglobulin production was not quite clear. In addition, each tested material is different in backbone and configuration patterns, therefore, the configuration concerning to its sulfate pattern, degree of sulfation and together with the structure of the backbone should be discussed. However, this is the first report studied the effect of sulfated polysaccharides on the immunoglobulin production of normal human PBMCs.

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