Effects of activated complements on platelets[1]

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KEY WORDS complement; blood platelets; zymosan; vascular endothelium; vascular smooth muscle

ABSTRACT

AIM: To observe the effects of activation of complements on platelets and subsequently on vascular endothelial cells (VEC) and vascular smooth muscle cells (VSMC). **METHODS:** Zymosan A-induced morphological change of platelets was determined with an aggregometer, and prothrombinase expression was quanitified using chromogenic substrate. Supernatant of zymosan A-treated platelet rich plasma (PRP) was added to the cultured VEC or VSMC for the observation of cell growth, DNA content, and the membrane microviscosity. **RESULTS:** Zymosan A induced marked metamorphosis, increased membrane microviscosity, and increased prothrombinase expression of platelets in PRP, but platelet metamorphosis induced by zymosan A was not found in washed platelets and fresh platelet suspended in platelet poor plasma (PPP) which had been pretreated with cobra venom factor (CVF). The effect of zymosan A on platelets was prevented by egtazic acid 5 mmol/L, Mn^{2+} 10 mmol/L, tetrodotoxin 40 μmol/L or indomethacin 100 μmol/L. The supernatant of zymosan A-treated PRP inhibited the growth of VEC, but accelerated the growth of VSMC and made most VSMC enter G_2- and M- phase. The endoplasmic reticulum with rough surface and free ribosome in VSMC and vacuoles appeared in VEC mitochondria. **CONCLUSION:** Activated complements induced significant shape change of platelets, stimulated platelets to release some factors and subsequently injured VEC, but accelerated the growth of VSMC, which may contribute to the development of blood coagulation and to the chronic inflammation.

INTRODUCTION

Activation of complements is a key response in the fight against infections and other immunological challenges. On the other hand, activated complements in many autoimmune and inflammatory diseases clearly induce reactions leading to tissue damage either directly by membrane attack complex (MAC) of complements or indirectly by leukocytes responding to the signals generated by complement activation. It has been reported that intravascular blood coagulation exists in complement-dependent injuries such as experimental respiratory distress syndrome, which may be the result of the interaction between complement-injured platelets and endothelial cells[1,2]. The vascular endothelial cells constitute a lining layer in the luminal side of blood vessel walls. This location is easy to be attacked by a lot of pathogens. As compared with other platelet aggregation inducers, cobra venom factor (CVF), a C3 and C5 converting enzyme with a long acting half-life time, can induce marked platelet metamorphosis, slow and slight aggregation, mild ATP release, and increased prothrombinase expression on the platelet surface[3,4]. These actions are complement-dependent and relative

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Received 2002-05-08 Accepted 2003-01-25

to the elevation in intracellular free calcium ions, and the reduction in cAMP[3,5]. In the present study, another complement activator, zymosan A[6,7], was used to understand the effect of complements on platelets and the effect of supernatant fluid from zymosan A-treated platelet rich plasma (PRP) on VEC and VSMC.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats weighing (300±50) g were provided by the Experimental Animal Center of Sun Yat-Sen University (Grade II, Certificate No 26-98A001). Fresh calf aorta was obtained from Guangzhou dairy.

Materials CVF was isolated and purified from the venom of south Chinese cobra (*Naja naja atra*) according to the method published previously[8], One unit of anticomplement activity of CVF was determined as 1.28 μg according to Vogel's method[9]. Zymosan A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and medium 199 were purchased from Sigma (USA); DMEM from Gibco-BRL (USA); trypsin, from Beijing Tianxiangren Biotech (China); fetal bovine serum, from Hangzhou Sijiqing Biotech-material (China); Factor VIII factor-related antigen, from DAKO (Denmark). All solvents and other reagents were of AR grade.

Preparation of PRP PRP was prepared from rat arterial blood using 3.8 % trisodium citrate as anticoagulant. Ten minutes after 150 ×*g* centrifugation, zymosan A was then added to PRP to a final concentration of 5 mg/L (the concentration which induced maximal action on platelet in PRP). After incubation at 37 ° C, it was centrifuged at 1000 ×*g* for 15 min, the platelets were discarded and the supernatant was stored at -20 ° C for available use. Washed platelets were prepared by chromatography on sepharose 2B column (2 cm×15 cm) eluted with Tris-HCl buffer (50 mmol/L, pH 7.2) containing NaCl 140 mmol/L, $MgCl_2$ 1 mmol/L, glucose 0.1 %, and bovine serum albumin 0.35 %. Finally, platelet count was adjusted to 2×10^8/L with the same buffer[10].

Determination of platelet metamorphosis Platelet metamorphosis was quantified photometrically as the maximal rate of change in light transmission of PRP or washed platelet suspension at 37 ° C under constant stirring using the Chrono-Log aggregometer (USA) connected to an IBM computer through Aggro/Link interface (Model 810). The prothrombinase activity was quantified according to the thrombin activity induced in the presence of prothrombin with the procedure described by Kawai[11].

Microviscosity measurement of cell membranes Fluorescence labeling and steady-state polarization measurements were performed with a Hitachi 850 spectrofluorometer[12], 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as fluorescence .

Culture of VEC and VSMC Under sterile condition, VEC were isolated from the bovine thoracic aorta and placed in pH 7.4 Hanks' balanced salt solution (HBSS). Surrounding fat and connective tissue were removed. The luminal surface was exposed and incubated in 0.125 % trypsin at 37 ° C for 15 min. Thereafter the luminal surface was gently flushed with Medium 199 supplemented with 20 % fetal bovine serum (FBS), benzylpenicillin 60 mg/L and streptomycin100 mg/L. The detached VEC were planted and seeded onto culture flasks. All VEC used in this study were taken between the 2nd-5th-passage culture. The endothelial cells were confirmed based on their growth in a regular monolayer, expression of endothelial cell-specific factor VIII was shown as yellow grains in the cytoplasm after immunohistochemical stain.

VSMC were obtained from the intimal-medial layers of the thoracic aorta from male Sprague-Dawley rat. The intimal-medial layers were cut into 1 mm^2 explants and VSMC grew in DMEM supplemented with 20 % fetal bovine serum, benzylpenicillin 60 mg /L, and streptomycin 100 mg/L, at 37 ° C in a humidified atmosphere of mixed gas of 5 % CO_2 and 95 % O_2. When explants reached confluence, they were trypsinized, centrifuged, and suspended in DMEM containing 10 % fetal bovine serum. The 4th-8th-passage cells were used for all growth studies. VSMC were identified by immunohistochemistry with mAb specific for α-smooth muscle actin and parallel myofilament shown in electron microscope (TEM, H-600, Hitachi, Japan).

Determination of cell growth In order to determine the cell viability, MTT 10 μL (5 g/L) was added to each well and incubated at 37 ° C for 4 h. The formazan product was solubilized with Me_2SO 100 μL. The absorbance of each well was measured using plate reader (Bio-Rad Model 3550) at 490 nm with reference at 630 nm.

Morphological examination of cells Determination of the ultrastructure of studied cells was carried out with TEM. The samples were washed with PBS, fixed for 4 h with 2.5 % glutaraldehyde in 0.1 mol/L

sodium cacodylate buffer (pH 7.2) at 37 °C, post-fixed with 1 % osmium tetroxide in the above buffer for 1 h, and dehydrated with increasing concentrations of ethanol-water mixtures. After embedment in epon resin 812, ultrathin sections were stained with uranyl acetate and lead citrate and were observed under TEM.

Preparation of flow cytometry of VSMC After incubation in 8 % supernatant of zymosan A-treated PRP for 24 h, the VSMC were treated with trypsin, centrifuged at 150 ×*g* for 3 min, carefully washed with PBS, filtered out higher order aggregates, and fixed in 70 % ethanol at 4 °C overnight. After being washed twice with PBS and stained with a solution containing propidium iodide 40-50 mg/L in the dark at 37 °C for 30 min, samples were analyzed in triplicates with a Flow cytometry (Beckman, USA) with excitation at 488 nm and emission at 610 nm.

RESULTS

Effect of zymosan A on platelets In the concentration range from 1 to 5 mg/L, zymosan A induced rat platelet metamorphosis in a concentration-dependent manner (Y=32.9 + 22.0X, X is the logarithmic concentration of zymosan A. Y is the decreased percentage of transmission, r=0.7835, P<0.01, n=12). Slight aggregation (less than 20 %) followed the metamorphosis. However, zymosan A 5 mg /L did not induce metamorphosis of washed platelets prepared with chromatography on sepharose 2B column. If fresh PPP was added to the washed platelets, metamorphosis appeared (Fig 1A). PPP pretreated with CVF 0.2 mmol/L for 15 min had no effect (Fig 1B). Zymosan A 5 mg/L-induced platelet metamorphosis was inhibited by egtazic acid 5 mmol/L or $MnCl_2$ 10 mmol/L (Fig 2A, B, C), indicating that this action of zymosan A was dependent on extracellular calcium ions. In addition, tetrodotoxin 40 μmol/L, or indomethacin 100 μmol/L also inhibited zymosan A-induced platelet metamorphosis, as shown in Fig 2 D, E.

After adding zymosan A 5 mg/L into PRP, the thrombin activities (relative to prothrombinase expression in platelet surface) at 0, 1, 2, 4, 8, and 16 min were (0.20±0.02) (n=9, as control), (0.35±0.02), (0.54±0.03), (0.42±0.03), (0.38±0.03), and (0.32±0.02) U (n=9, P<0.01 *vs* control), respectively.

Zymosan A 1-5 mg/L increased membrane microviscosity (η) of platelets in a concentration-dependent manner (Y=9.28+8.15X, X: the logarithmic concentration of zymosan A. Y is the increased percentage of membrane microviscosity. r=0.986, P<0.01, n=12).

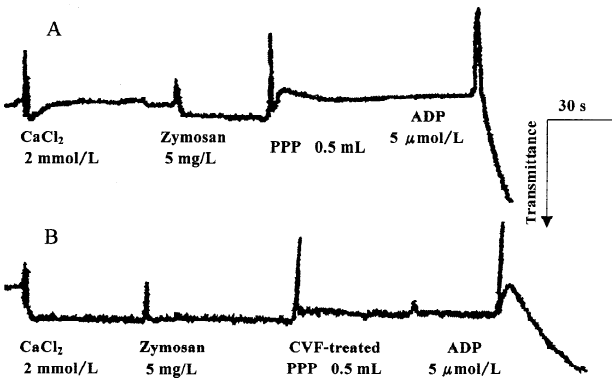

Fig 1. Effect of zymosan A 5 mg/L on washed platelets prepared with chromatography on sepharose 2B column. Zymosan A did not induce metamorphosis of washed platelets. Metamorphosis appeared if fresh PPP was added (A) but not PPP pretreated with CVF for 10 min (B).

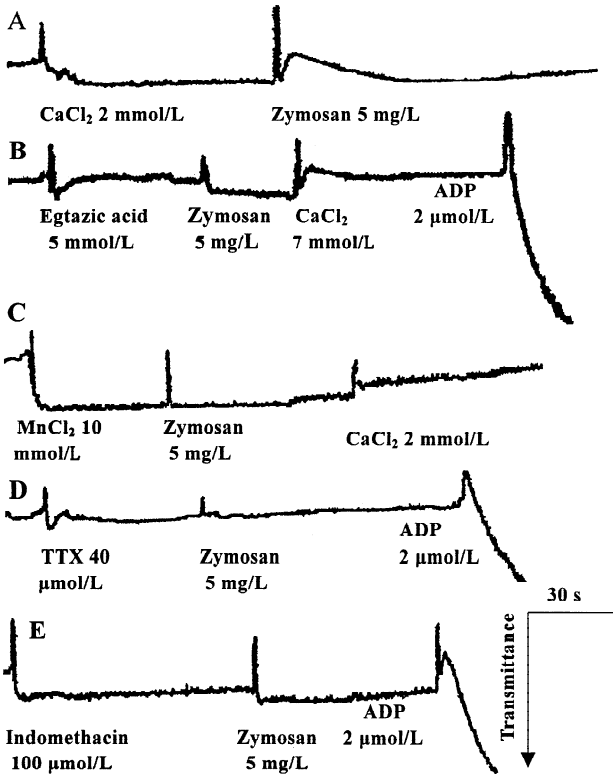

Fig 2. Zymosan A induced metamorphosis of washed platelets prepared with chromatography on sepharose 2B column. A: Zymosan A induced metamorphosis in the presence of Ca^{2+}; the inhibitory effects of egtazic acid (B), Mn^{2+} (C), tetrodotoxin (D), and indomethacin (E) on it.

Supernatant of zymosan A-treated PRP inhibited the growth of vascular endothelial cells Super-

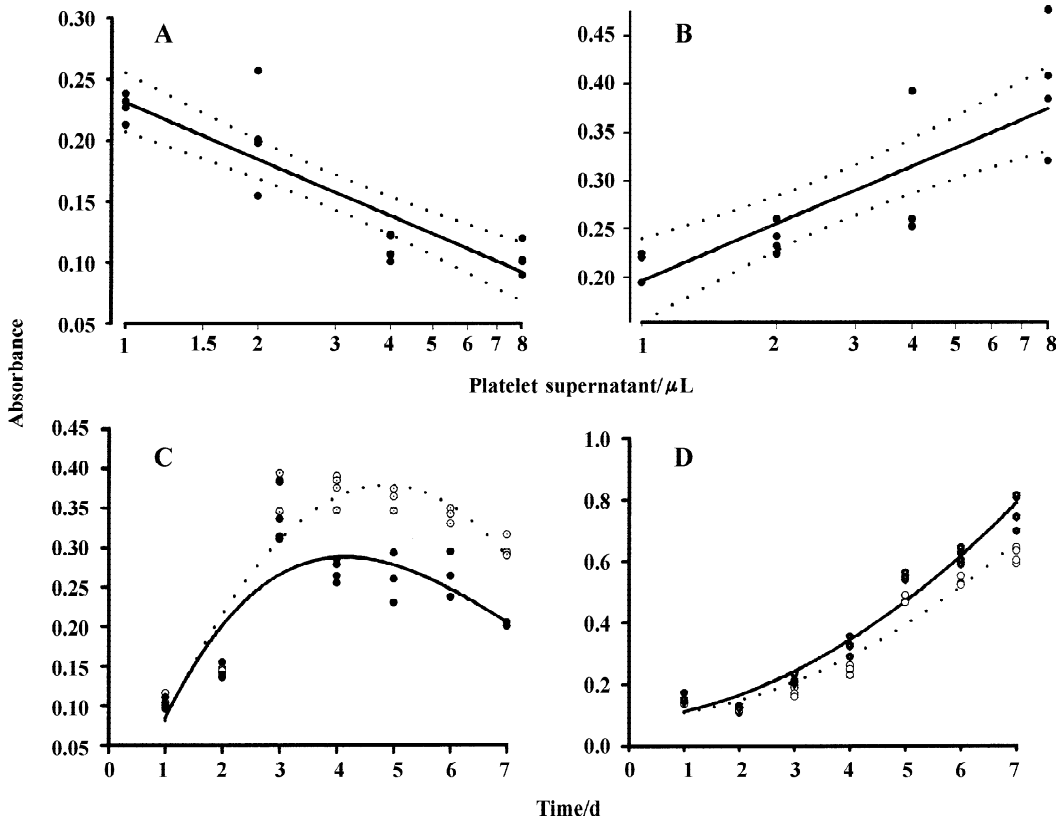

Fig 3. Effect of zymosan A-treated PRP on the growth of VEC and VSMC. The vital cells were determined by MTT method. The absorbance reflexes the vital cell count. A: Dose-effect relationship between zymosan A-treated-PRP and vital VEC counts. B: Dose-effect relationship between zymosan A-treated-PRP and vital VSMC counts. Black line is regressive line, dash lines are the 95 % confidence limits. C: The growth curve of VEC. D: The growth curve of vital VSMC. ●: Vital cell counts after addition of 1 % zymosan A-treat-PRP; black line is its regression line. ○: Vital cell counts as control; dash line is its regression line. Significant differences between two groups were found at d 4, d 5, d 6, and d 7 (P<0.01; t-test, n=8) in vital VEC counts; at d 6 and d 7 (P<0.05; t-test, n=8) in vital VSMC counts.

natant from zymosan A-treated PRP injured cultured VEC in a concentration-dependent manner (Y=0.231-0.154X, X: the logarithmic concentration of zymosan A, Y : the decreased percentage of absorbance, r= -0.7984, P<0.01, n=16, Fig 3A). After 1 % (v/v) supernatant of zymosan A-treated PRP was added to the cell culture medium, once a day for 7 d, the vital cell counts of VEC decreased significantly as compared with control. The vital cell counts (absorbance unit) of VEC in control group (n=4) at d 4, 5, 6, and 7 were 0.37±0.09, 0.36±0.01, 0.34±0.01, and 0.30±0.01 respectively, while those in experiment group (n=4) at the same time were 0.27 ±0.01, 0.25±0.02, 0.26±0.01, and 0.20±0.01. The time-cumulative effect relationship was shown in Fig 3C. Under the TEM, organelles decreased, pinosome disappeared, and mitochondrion vacuoles appeared in injured endothelial cells (Fig 4A).

Supernatant of zymosan A-treated PRP accelerated the growth of vascular smooth muscle cells Supernatant of zymosan A-treated PRP accelerated the growth of cultured VSMC in a concentration-dependent manner (Y=0.196+0.199X, X is the logarithmic concentration of zymosan A, Y is the increased percentage of absorbance, r=0.668, P<0.01, n=16, Fig 3B). After 1 % (v/v) supernatant of PRP pretreated with zymosan A was added to the cell culture medium, once a day for 7 d, the vital cell counts of VSMC increased significantly as compared with control. The vital cell counts (absorbance unit) of VSMC in control group (n=4) at d 6 and d 7 were 0.55±0.02 and 0.59±0.01, while those in treatment group at the same time were 0.62±0.01 (P <0.05 *vs* control) and 0.78±0.04(P<0.01 *vs* control) respectively. The time-cumulative effect relationship was shown in Fig 3D. Under the TEM, VSMC showed rough surfaced endoplasmic reticulum (Fig 4C).

Cell cycle analysis indicated that the DNA content

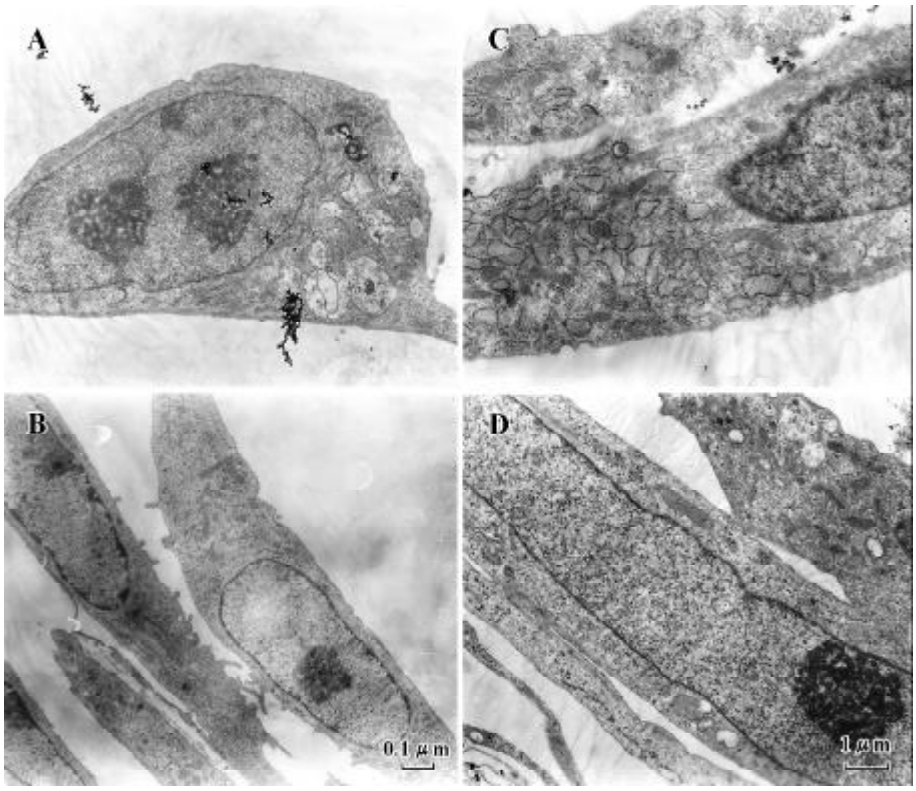

Fig 4. Microphotograph of cultured endothelial cells (×60 000) and smooth muscle cells (×8000) influenced by supernatant of zymosan A-treated PRP. A: Injured endothelial cells by 8 % supernatant of zymosan A-treated PRP for 12 h. B: Normal endothelial cells. C: Proliferous smooth muscle cells by 8 % supernatant of zymosan A-treated PRP for 24 h. D: Normal smooth muscle cells.

of VSMC, after addition of supernatant of zymosan A-treated PRP for 24 h, decreased from 88.9 % to 69.2 % in G_0 phases, increased from 6.0 % to 11.6 % in G_2 phases, and increased from 5.1 % to 18.9 % in S phase, respectively. Therefore it was considered that supernatant of zymosan A-treated PRP enhanced proliferation of VSMC.

DISCUSSION

The present studies demonstrate that treatment of PRP with zymosan A results in the change of platelets through activation of complements, and there are materials released from platelets which enhance the proliferation of VSMC, whereas it is important in response to injury of VEC.

Activation of plasma complements initiates blood platelets morphological and functional changes[3]. Cobra venom factor (CVF) is a long acting complement activator resulting in exhaustion of complements, so that it is otherwise named anti-complementary protein. Zymosan A, a complex polysaccharide obtained from *Saccharomyces cerevisiae* (*Escherichia coli*), is a complement activator including activation of the terminal complement components, predominantly through the alternative pathway[6]. These two substances were used in this study as tool agents to investigate the pathogenesis potential induced by activated platelets from both positive and negative sides.

The role of platelets in blood clotting and the role of complements in immune reaction were accepted [6], but links between these two factors and their subsequent effects remain to be investigated.

One result of present study indicates that complement activation induces metamorphosis followed by slight aggregation of platelet, but platelet-released active substances were not identified yet. On the other hand, when plasma complements were exhausted by CVF or absent in the case of washed platelet preparation, platelets remained unaffected.

Anyhow, active substances released in the supernatant fluid of zymosan A-treated PRP are evidenced by suppressing growth of vascular endothelial cells and promoting proliferation of vascular smooth cells.

As a whole, this study provides gross evidences that pathological activation of platelets may subsequently induce vascular endothelial damage, proliferation of vascular smooth cells, intravascular thrombosis and possibly, in a long run, atherosclerosis.

In conclusion, factors activating complements trigger platelet activation, and subsequently initiate a cascade of pathogenetic changes and finally result in vascular endothelial damage, proliferation of vascular smooth cells, intravascular thrombosis and possibly, in a long run, atherogenesis. The results of this study also indirectly show that anticomplement drugs might be useful to prevent and/or to treat atherosclerosis diseases. In fact, CD59, has been shown to be effective in preventing the hyperacute phase of rejection appeared in xenotransplantation[13]. While a soluble complement receptor 1(CR1) molecule, produced by recombinant DNA technology, also prevented ischemia/reperfusion injury, thermal trauma, and immune complex mediated inflammation[14,15].

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