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行政院國家科學委員會專題研究計劃成果報告

敗血症治病因和治療之研究--比較格蘭式陰性及陽性菌毒素對刺激小神經膠細胞、白血球及血小板釋放cytokines及nitric oxide的相對活性

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一、中文摘要

至目前為止，一般相信格蘭氏陰性菌(gram-negative) 及其內毒素lipopolysaccharide (LPS) 是造成敗血症的主要原因；較常見且具高致命性的反應如引起低血壓，血小板減少症，瀰漫性血管內凝血以及最後常導致許多器官的衰竭而死亡。另一方面，在最近幾年的研究中亦發現格蘭氏陽性菌 (gram-positive) 所引起的細菌感染亦能引發全身性的細菌感染與敗血性休克；據統計約有1/3到1/2的敗血症是由陽性菌所引起的，並且相信在未來的幾年中其所占的比例還會再繼續增加。引發敗血症的過程目前被認為是因許多細胞被活化產生 cytokines (如 IL-1β, PAF, TNFα 等)的結果；主要參與這些反應的細胞如單核球 (monocyte), 淋巴球 (lymphocyte), 嗜中性白血球 (PMN) 及血小板 (platelet) 等；在中樞神經系統中，最明顯的反應是小神經膠細胞 (microglia) 的活化；此細胞不論在細胞型態，免疫表型或生理功能上都與單核球/巨噬細胞 (macrophage) 相似。

研究陽性菌引發敗血症最困難的一點在於不同的菌種間其細胞壁的成份各有不同。其中lipoteichoic acid (LTA) 為陽性菌細胞壁中最重要的成份之一，目前被認為在陽性菌引起敗血症的過程中， LTA 扮演非常重要的角色。

因此本計畫的重點主要在探討及比較陰性菌的主要內毒素LPS和陽性菌的LTA對於 microglia, 各種白血球以及對血小板的作用差異性；並由其中的實驗可瞭解LPS和LTA的相對作用強度及個別作用機轉。

關鍵詞:LTA, 小神經膠細胞，血小板

Abstract

At present, it is a widely believed that sepsis in caused predominantly by gram-negative organisms, and endotoxin LPS (lipopolysaccharide), a substance produced by these organisms. The progression of sepsis is cardiovascular dysfunction (i.e., hypotension, thrombocytopenia, disseminated intravascular coagulation) and multiple organ by dysfunction syndrome (MODS), finally associated with an increase in the mortality. Up to now, the important of gram-negative organisms in the genesis of sepsis has been emphasized. However, recent studies show an increasing evidenced of gram-positive sources of sepsis. On the basis of this evidence, it seems reasonable to conclude that between one third and one half of all cases of sepsis are currently caused by gram-positive organisms and that the incidence of gram-positive sepsis should continue to rise for at least the nest few years. Sepsis is believed to result from a complex mechanism involving activation of a number of cells, most notably monocytes, lymphocytes, neutrophils and platelets as well as microglia. Microglia are like macrophages in that they are derived from mononuclear myeloid progenitors, they

reside in the CNS in a ramified, quiescent states, but can readily migrate to sites of inflammation in the CNS.

One of the chief difficulties in elucidating how gram-positive organisms cause sepsis is that they are considerable differences in cell wall composition among various gram-positive species. Lipoteichoic acid (LTA), a predominant component associated with the cell wall of gram-positive bacteria, can provoke marked stimulation of sepsis.

The present project is designed to compare the relative activities of LPS and LTA in stimulation of cytokines (i.e., IL-1β, TNFα) and NO release in microglia, monocytes, lymphocytes, neurophils and platelets in vitro and ex vivo, respectively.

Key words: LTA, microglia, platelet

二、緣由與目的

細菌感染(特別是陰性菌)常引起敗血性休克(septic shock)；而臨床上的病徵常因感染的時期不同和發病過程不同而有不同的臨床病徵表現(Bone, 1991)。較常見且具高致命性的反應如引起低血壓；因微血管通透增加而引起的滲漏(microvascular leaky), 心肌失去功能 (myocardial dysfunction)，瀰漫性血管內凝血(DIC, disseminated intravascular coagulation)等；最後，常導致許多體內器官的衰竭如腎皮質壞死 (renal cortical necrosis) 而死亡 (Parrilo et al., 1990)。雖然引起敗血性休克的病理因素非常複雜，但目前已知寄主的發炎反應乃是造成休克及多重器官衰竭的主要原因 (Dunn, 1991; Raij et al., 1977)。細菌體特別是格蘭氏陰性菌 (gram-negative) 其細胞壁外層之成份LPS (lipopolysaccharide) 為其主要的毒性來源 (Dunn, 1991), 會刺激寄主而引起許多發炎媒介物的產生；包括各種不同的cytokines (如腫瘤壞死因子, TNFα), prostaglandins, platelet activating factor (PAF)及 kinins等等 (Dunn, 1991)。當這些發炎物質累積到足夠量時，便會釋放進入全身循環中而影響到如心臟、血管等主要器官 (Gaynor, 1970)。至於LPS是透過何種次級媒介物 (secondary mediator) 而造成體內如此複雜的病理現象，則目前尚未完全清楚。若能進一步確認這些媒介物的角色及其引發的可能作用機轉，則將有助於瞭解敗血症之種種不同的病理作用。

雖然幾年對於格蘭氏陰性菌所引起的敗血性休克已獲得廣泛的研究與證實，然而對於格蘭氏陽性菌所引起的敗血性休克則已開始被大家所重視。這幾年研究發現由格蘭氏陽性菌所引起的細菌感染亦能引發全身性細菌感染與敗血性休克(Bone, 1994)。據統計約有1/3到1/2的敗血症是由陽性菌所引起的，而且在未來的幾年中還會繼續的增加。因為陰性菌之內毒素(如LPS) 為單一成份較易純化，因此較易引起醫學界的注意；然而陽性菌因含有許多不同的成份，因此遲至1980年代左右，學者才開始注意到陽性菌在敗血症過程中所扮演的角色 (Bone, 1994)。

研究陽性菌最困難的一點是在不同的菌種間其細胞壁的成份各有不同。其中lipoteichoic acid (LTA) 為陽性菌的細胞壁中最重要的成份之一，在細菌溶解時會被釋放出來；LTA主要由甘油所組成，一端結合在細胞膜的磷脂上，另一端則形成甘油-磷酸壁 (teichoic acid) 聚合體，穿過peptidoglycan layers而成為細胞壁成份之一。由於LTA帶有陰性電荷(磷酸基)，故可連結及調節陽離子之進出細胞；另外，LTA在細菌生長時亦擔任重要角色。最近的研究顯示LTA在臨床實驗上可對動物及人體造成發熱、白血球減少 (leukocytopenia)、低血壓 (hypotension)、體內器官受損、血小板減少症 (thrombocytopenia), 瀰漫性血管內凝血 (disseminated intravascular coagulation, DIC)等相關敗血性休克的病理現象 (Bone, 1994)。

在敗血性休克過程中有較高致命性

反應的症狀:如引起低血壓、微血管通透性增加、心血管失去功能(cardiovascular dysfunction), 瀰漫性血管內凝血以及導致體內器官的衰竭而死亡等等(Danner et al., 1991),由陽性菌所造成的機率可能會更高(Bone, 1994)。目前已知LTA會刺激體內許多細胞(如單核球，嗜中性球及淋巴球)而引起許多發炎媒介物的產生；包括一些 cytokines [如 interleukin-1β (IL-1β), IL-2, IL-6, IL-8, TNFα (tumor necrosis factor α), PAF]等, thromboxane, prostagladins, proteases以及free O_2 radicals等等(Bhakdi et al., 1991)。至於LTA是如何造成如此複雜的病理現象，至今尚未完全清楚；若能進一步去確認並了解其引發的可能作用機轉，則有助於提供臨床上治療陽性菌所引起的敗血性休克一個新的治療方向。因此，本計畫擬在此方面作一詳加探討。

另一方面，在陽性菌感染引起敗血症的過程中，其中一項明顯的變化為造成血小板減少症 (thrombocytopenia)；其所造成的原因是否與陰性菌感染是一樣的，目前不得而知。雖然血小板的生合成可能造成一部份的原因；但最重要的原因可能是增加血小板在體內的消耗有關。LTA對血小板的作用於1977年便有學者指出: Streptococcus的LTA在in vitro的實驗中會抑制血小板的凝集反應(Beachey et al., 1977)；在1990年亦學者發現Staphylococcus之LTA會影響fibrin與platelet之間的附著(Chugh et al., 1990)；至於LTA對於血小板的詳細作用機轉至今尚未有完整的報告；因此本計畫擬進一步探討陽性菌對血小板的作用及機轉的分析。

中樞神經系統主要由神經細胞及其突起和一系列特化的支持細胞所構成；這些非神經的支持細胞如星狀細胞 (astrocyte), 寡樹突膠質細胞 (oligodendrocyte), 室管膜 (ependyma) 和小神經膠細胞(microglia)統稱為神經膠細胞 (neuroglia)。在正常中樞神經系統內，microglia 占全數神經膠細胞族群的5~20 %。這些細胞普遍分布在中樞神經系統，而且依據它們所處的區域不同，有不同的外形及細胞密度。Lawson 等人 (1990) 發現 microglia 在成熟小鼠腦分佈發現小神經膠細胞在海馬迴、嗅覺皮質、基底核及黑質有較高的分佈、而在神經徑束上含較小的族群密度。一般而言，根據 microglia 在神經組織所在位置，可分兩種類型，即血管旁的小神經膠細胞 (perivascular cell) 和近血管的小神經膠細胞 (juxtavascular microglia)，其角色似靜止的小神經膠細胞 (resident microglia)。然而不論是那種 microglia, 皆在細胞型態，免疫表型和生理功能上和白血球 (monocyte)/巨噬細胞 (macrophage) 相似 (Flaris et al., 1993)。所以一般相信 microglia 源自於血液中的大單核白血球經由中間的過度細胞-變形性小神經膠細胞 (amoeboid microglial cell), 後者在出生前後的腦組織內擔任巨噬細胞的功能。然而仍有些學者宣稱 microglia 是來自神經外胚層 (neuroectoderm) (Ling et al., 1993)。這些矛盾的說法可能導因於不同的研究方法的使用以及缺乏一項專一且可信度高的標誌物來標誌 microglia。最近免疫細胞化學的研究顯示，microglia 在不同的神經病變中會活化成腦巨噬細胞 (brain macrophage) 執行吞噬功能，此時表面抗原像 Fc 及 CR3 受體會增加，且伴隨快速誘導組織相容第一型及第二型抗原 (MHC class I & II antigens) 的新合成 (Streit et al., 1989)。所以毫無疑問的 microglia 有吞噬及免疫的潛能。當中樞神經系統受傷時，microglia 會活化而作為第一道防禦線。活化過程包括細胞增生、增加免疫表型、microglia 補充到受傷區域 (Lindholm et al., 1992) 和功能改變，例如釋放NO等毒性物質或cytokines 等發炎物質 (如IL-1β, TNFα等) (Banati et al., 1993)。除此之外，microglia 有很強的抗原表現和明顯的毒性作用。再者，microglia 的活化呈階段性反應；如起初並沒有吞噬作用，過一段時間，特別是當神經或突觸末端退化時，才會轉變成腦內的吞噬細胞 (Streit et al., 1988)。在許多發炎反應或敗血症發生的過

程中，microglia 會釋放好幾種cytokines包括IL-1, IL-6, TNFα 和TGFβ (transforming growth factor β) 等(Dickson et al., 1993)；這些cytokines 都有助於星狀細胞的活化，誘導細胞附著分子(adhesion molecules)表現，增加T細胞到受傷區；並且在發炎後期會負責調節使疾病不至於惡化。同時，microglia 會釋放其它傷害物質，像是蛋白分解酵素(protease), 反應氧中間物(reactive oxygen intermediates)和一氧化氮 (NO) (Thery et al., 1991)。總之，中樞神經系統具有其獨特的免疫細胞 (immune cell) 群，主要以 microglia 為主，其為一種特化的巨噬細胞；當在中樞神經系統受傷時會表現出階段性的反應，而功能就好像是神經系統的偵測器。

當中樞神經系統發炎或發生敗血症時，microglia會移動到發炎的地區，釋放cytokines或其它發炎的物質。因此microglia對神經組織的修補和發育扮演一重要的角色，但若其過度活化，也會造成傷害。因此，本計畫擬針對陰性菌的LPS及陽性菌的LTA探討及比較此兩者對microglia釋放cytokines (如IL-1β, TNFα等)或NO的差異性以及表現nNO synthase (ncNOS)及 iNO synthase (iNOS) 的作用。

三、結果與討論

本研究結果顯示，在小鼠的小神經膠細胞 (microglia) 培養液中(5 x 10^6/ml)，單獨加入 LTA (之5-20 μg/ml) 很難促使小神經膠細胞 (microglia) 釋放 superoxide free radical (O_2^-), 即使濃度增加至50 μg/ml亦然；但若合併 LTA (5, 10 and 20 μg/ml) 與 protein kinase C activator, PMA (400 nM) 則可明顯的刺激小神經膠細胞釋放 superoxide free radical (O_2^-) (Fig. 1)。此釋放 free radical (O_2^-) 的反應亦隨著 LTA 加入的時間增加而增加 (數據未列出)；另外，加入 protein kinase C 的受體活化劑如 fMLP (100 nM) 亦可明顯的促進 superoxide free radical (O_2^-) 的釋放(Fig. 1)；由本研究顯示，LTA 的確可刺激小神經膠細胞釋放 superoxide free radical (O_2^-)；至於其更詳細的作用機轉，我們將繼續進一步研究。另外，是否 LTA 可抑制 NO 及 NO synthase 的表現，我們亦將進一步探討；利用 RT-PCR 探討是否 LTA 可抑制小神經膠細胞 NO synthase 的 mRNA 表現；以及利用 EMSA 來探討何種 transcription factor 負責調控 NO synthase 的 DNA 表現。

本計劃的另一個實驗為探討 LTA 對血小板凝集的影響；由結果顯示，LTA 可明顯的抑制血小板凝集反應；此抑制血小板凝集反應不只隨著 LTA 加入的濃度增加而增加 (0.1-10 ng/ml) (Fig. 2)，同時亦隨著反應的時間增加而增加 (10-60 min)，且在60 min達到最大的抑制效果 (Fig. 3)。在人類的血小板懸浮液中，LTA 可明顯的抑制 collagen (1 μg/ml), thrombin (0.05 U/ml) 及 ADP (20μM) 所引起的血小板凝集反應及 ATP 的釋放反應(Fig. 2)； LTA 抑制上述活化劑所需的 IC_{50} 分別為 0.7, 0.82 及 1.41 (ng/ml). 另外，單獨加入 LTA 並不會引起血小板凝集反應。

由上述的結果暗示著，LTA 的作用機轉可能不是經由作用在各個活化劑的受體位置上，而可能是作用在某個共同步驟上。因 LTA 本身是一種毒素，因此我們懷疑是否作用在血小板的細胞膜上而抑制血小板凝集反應。利用螢光物質 DPH (diphenylhexatriene) 標定血小板細胞膜，以便測定細胞膜的流動性；由實驗結果顯示，LTA (0.5 and 1.0 μg/ml) 隨著濃度的增加而增加對細胞膜流動性的影響 (Fig. 4)。進一步研究LTA的抑制機轉，我們發現LTA (0.5和1.0 μg/ml) 可明顯的抑制由 collagen 所引起的 phosphoinositide breakdown (Fig. 5)，以及細胞內鈣離子濃度的增加 (Fig. 6)；同時LTA亦能抑制collagen所引起的thromboxane A_2的形成；在47-kDa蛋白的磷酸化實驗中，可清楚瞭解LTA亦能明顯的抑制由PDBu所引起的

47-kDa蛋白質的磷酸化反應(Fig. 7)。

由以上的研究結果顯示，LTA抑制血小板凝集反應的作用機轉為先引起血小板細胞膜流動性的改變，進而抑制phospholipase C的活性，此結果導致phosphoinositide的分解減少及thromboxane A_2的合成降低，進一步引起細胞內鈣離子濃度的下降和47-kDa蛋白質磷酸化的抑制。

四、計畫成果自評

本研究計劃目前已達95 %當初申請計劃時的內容；且本結果我們亦正準備投稿在國際著名學術期刊。因此，我們算是達到當初所設定的目標。

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Table 1. Effect of LTA on the thromboxane B_2 formation in washed human platelets induced by collagen.

	Dose (μg/ml)	Thromboxane B_2 (ng/ml)
resting	____	26.7 ± 3.0
collagen	1	57.1 ± 0.6*
+ LTA	0.5	50.5 ± 0.5#
	1	34.5 ± 0.5#

Platelet suspensions were preincubated with LTA (0.5 and 1.0 μg/ml) for 10 min at 37℃, and then collagen (1 μg/ml) was added to trigger thromboxane B_2 formation. Data are presented as means ± S.E.M. (n=4). * $P < 0.001$ as compared with the resting group; # $P < 0.001$ as compared with the collagen group.

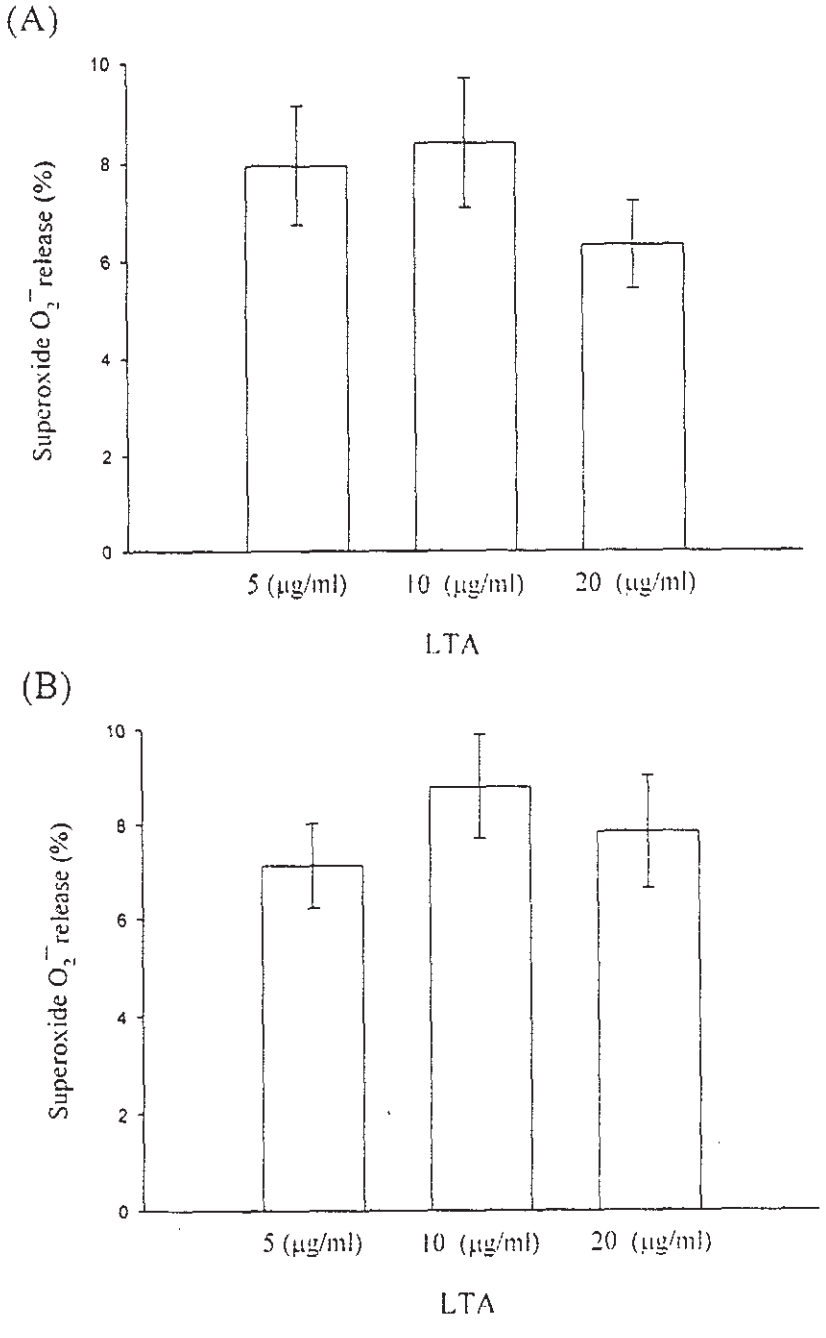


Fig. 1 Effect of LTA-induced superoxide free radical (O_2^-) formation in the presence of (A) PMA and (B) fMLP in culture microglia cells. Microglia cells (1.2×10^5/ml) were pretreated with various concentrations of LTA (5, 10, and 20 μg/ml) in the presence PMA (400 nM) or fMLP (100 nM) for 60 min. The mixtures were centrifuged (10,000 r.p.m.) for 20 min at 25 ℃, and the supernatants were then collected for superoxide free radical (O_2^-) assay. Data are presented as percent control (solvent, PBS) by mean ± S.E.M. (n=4).

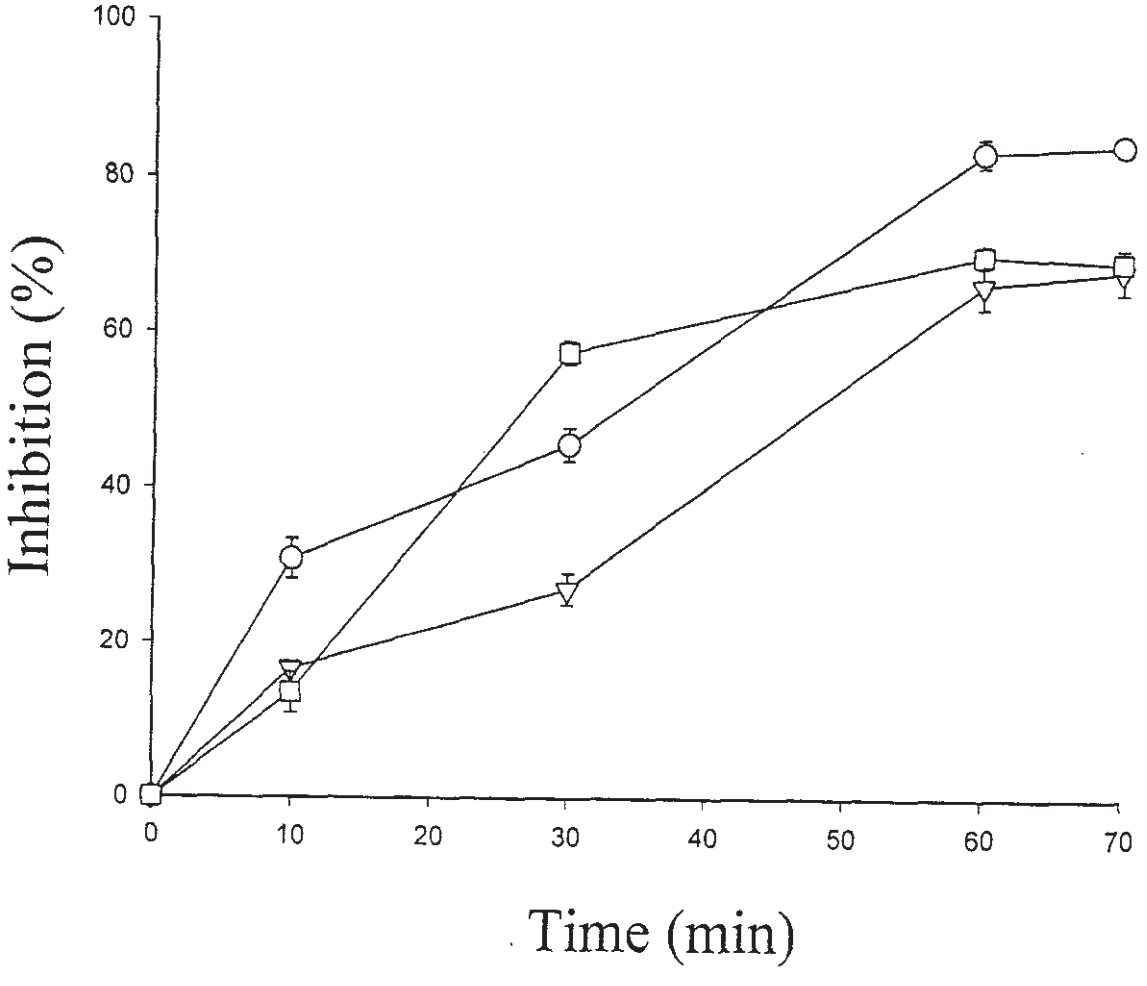
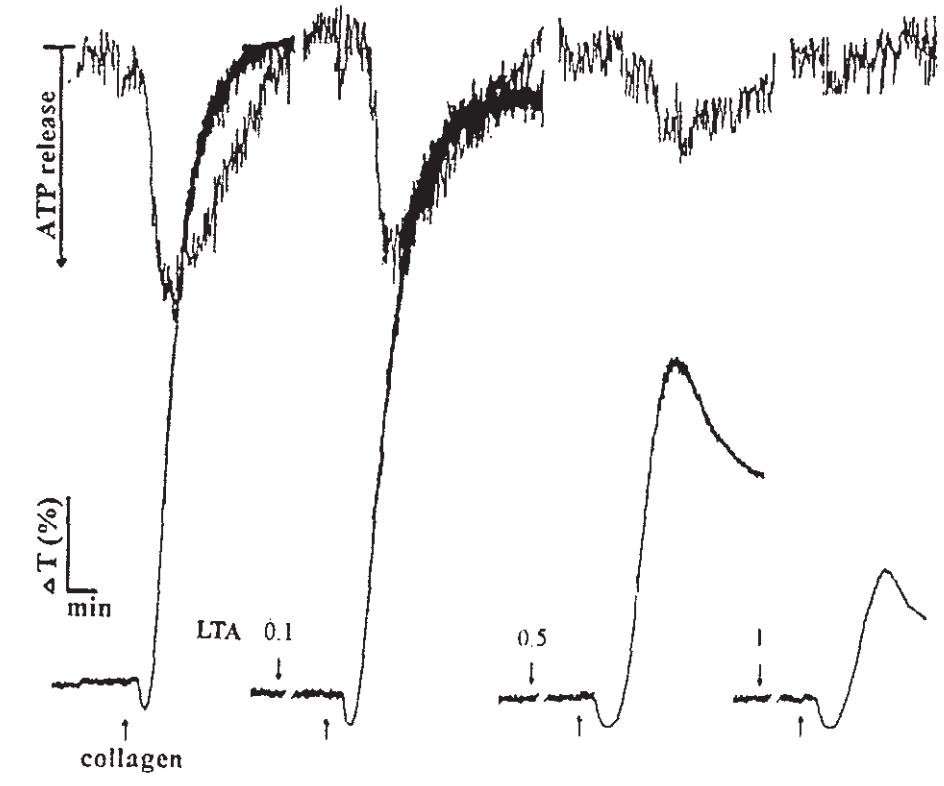


Fig 2. (A) Tracing curves of LTA on collagen (1 μg/ml)-induced aggregation and (B) dose-inhibition curve of LTA on collagen (1 μg/ml, ○)-, thrombin (0.05 U/ml, ▽)-, and ADP (10 μM, □)-induced platelet aggregation in washed human platelet suspensions. Platelets were preincubated with LTA (0.1-1.0 μg/ml) and stirred for 10 min, then agonists were added to trigger aggregation (upward tracings) and ATP release (downward tracings) (A). Luciferin-luciferase mixture (20 μl) was added 1 min before the agonist in order to measure the ATP-release reaction. Data are presented as percentage of control (means ± S.E.M., n = 4).

A



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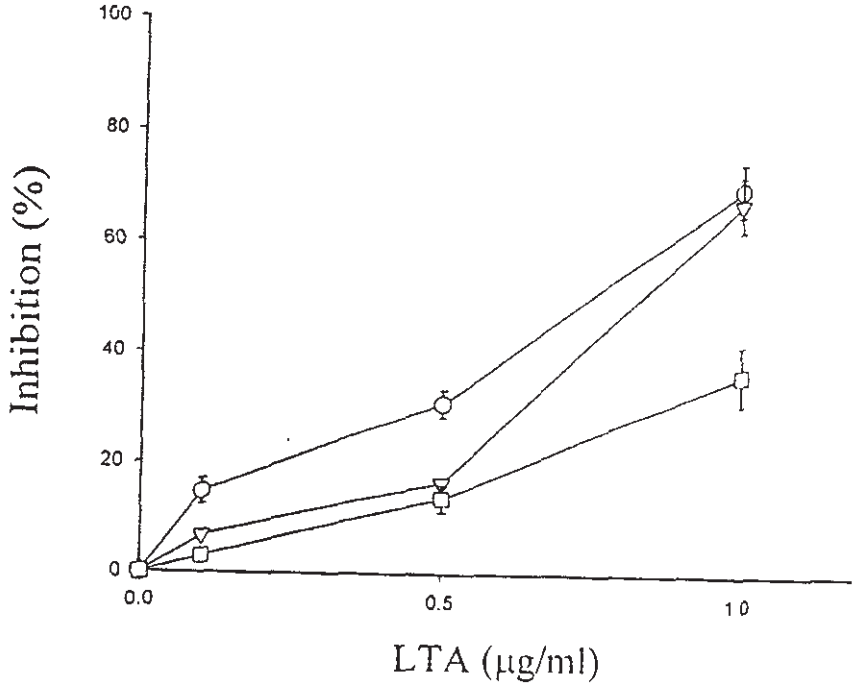


Fig 3. Time-inhibition curves of LTA on collagen (1 μg/ml, ○)-, thrombin (0.05 U/ml, ▽)-, and ADP (10 μM, □)-induced platelet aggregation in human platelet suspensions. Platelets were preincubated with LTA (0.5 μg/ml) and incubated for 10-60 min at 37℃, respectively. Aggregation agonists were then added to trigger aggregation. Data are presented as a percentage of control (means ± S.E.M., n = 4).

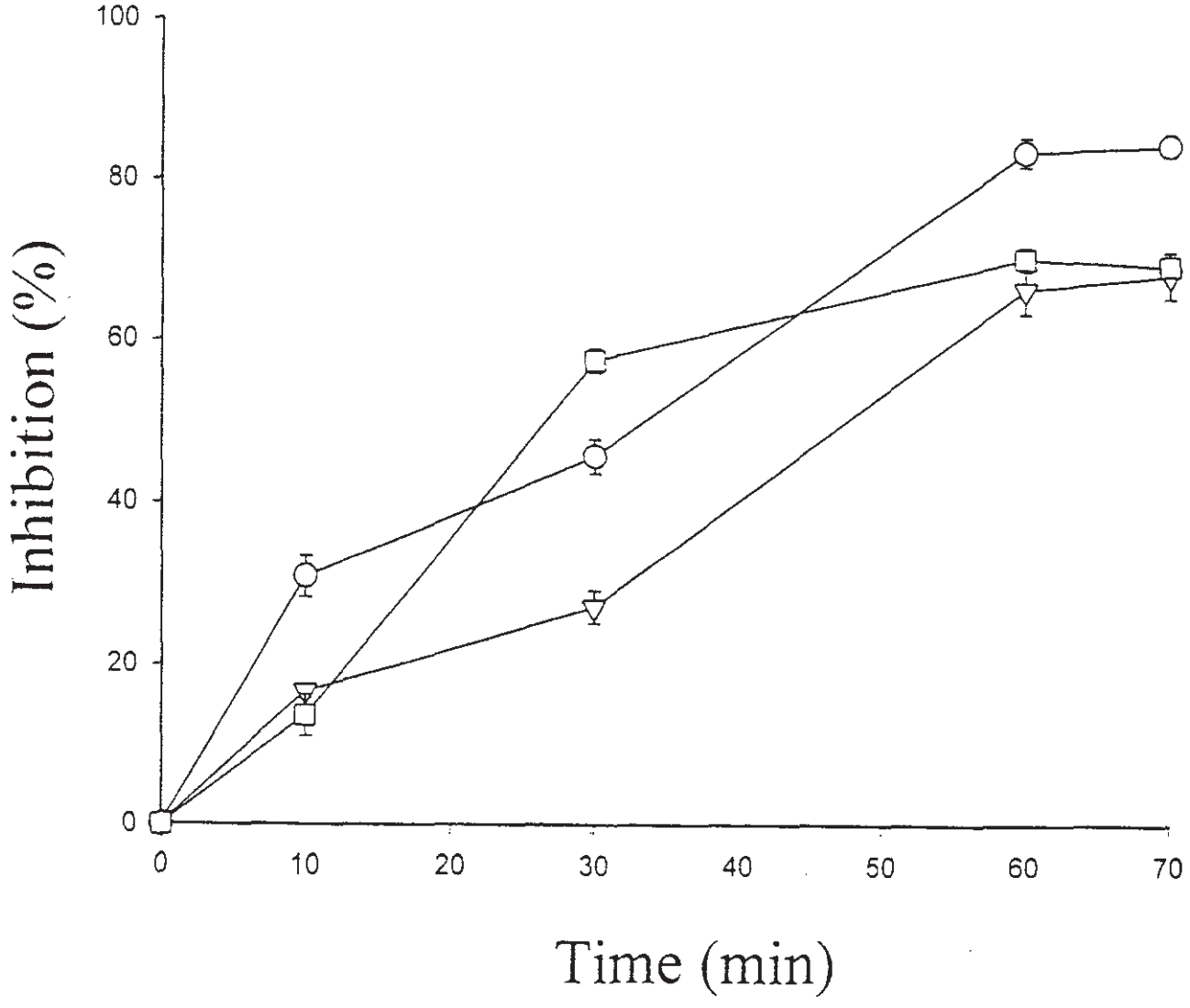


Fig 4. Fluorescence emission spectra of platelet membranes in the (A) absence or (B) presence of DPH (1 μM). Curves C and D are the emission spectra of membranes labeled with DPH in the presence of LTA (C) (0.5 μg/ml) and (D) (1.0 μg/ml) for 10 min. The profiles are representative examples of four similar experiments.

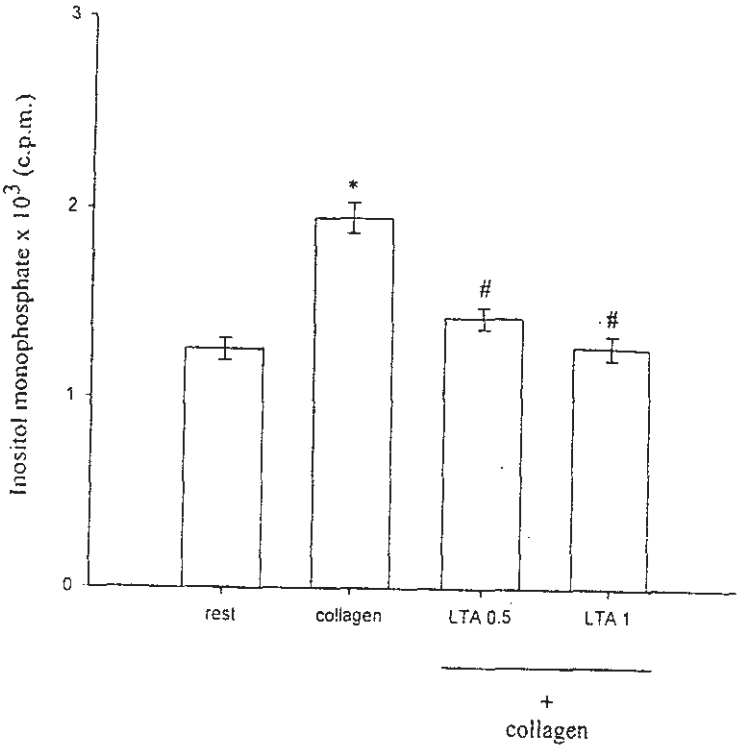


Fig 5. Effect of LTA on collagen-induced inositol monophosphate formation in human platelet suspensions. Platelets were labeled with [^{3}H]-inositol and stimulated with or without collagen (5 μg/ml) in the presence of LTA (0.5 and 1.0 μg/ml). Data are presented as means ±S.E.M. (n = 5). * $P < 0.001$ as compared with the resting group; $^\#$ $P < 0.001$ as compared with the collagen group.

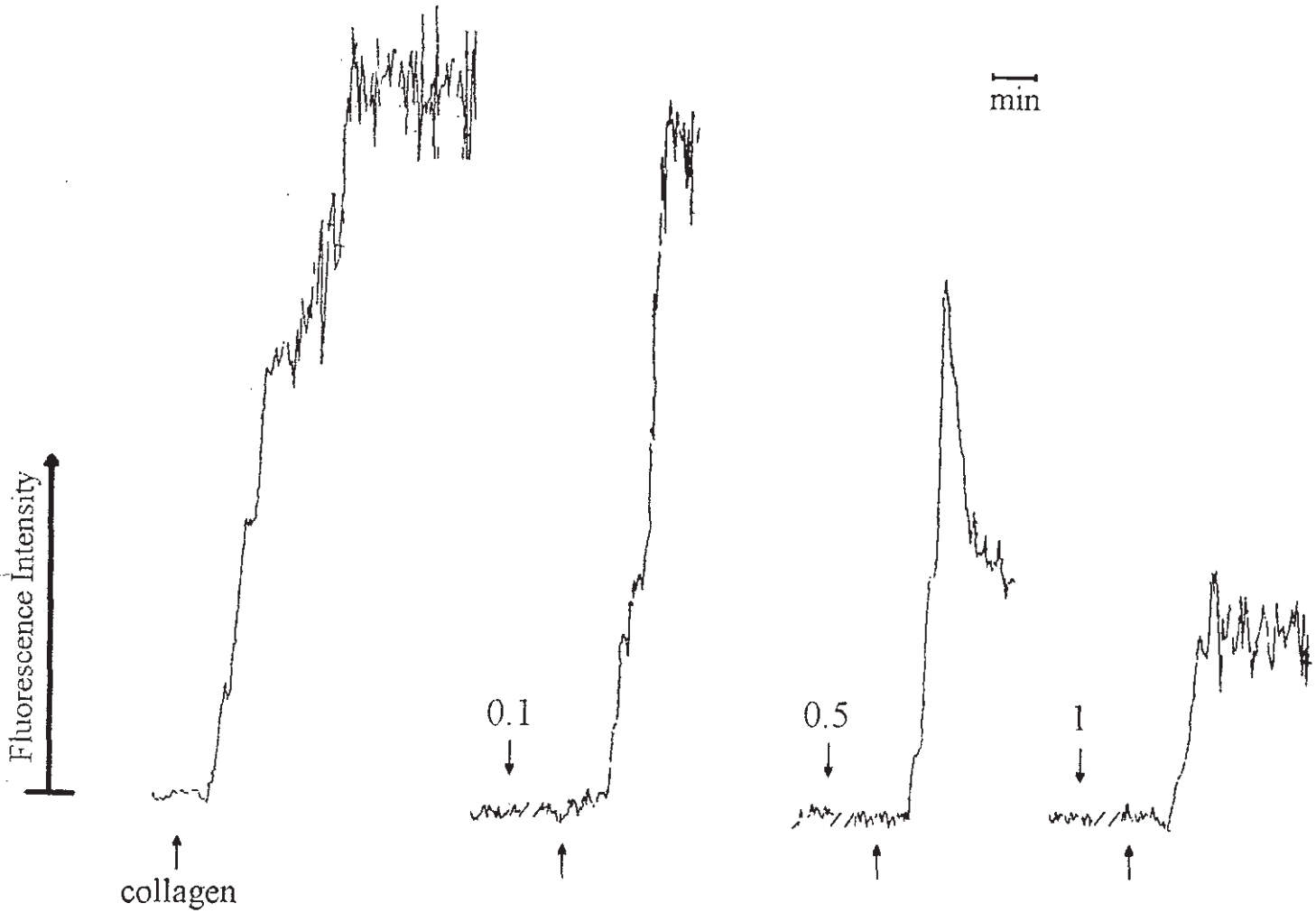
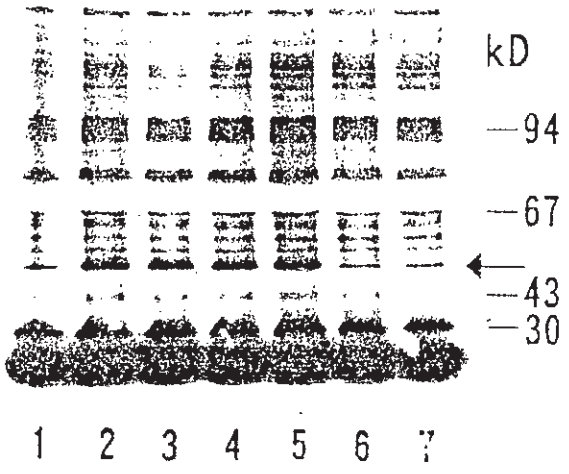


Fig 6. Effect of LTA on collagen-induced intracellular Ca^{2+} mobilization in Fura 2-AM-loaded human platelets. Platelet suspensions were preincubated with Fura 2-AM (5 μM) at 37℃ for 30 min, followed by the addition of collagen (1 μg/ml) in the absence or presence of LTA (0.1, 0.5, and 1.0 μg/ml), which was added 10 min prior to the addition of collagen (1 μg/ml).

A



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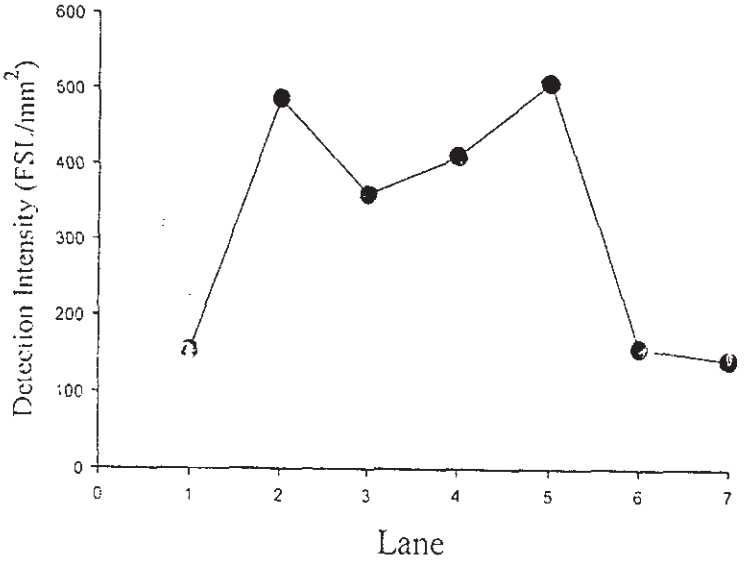

Fig 7. (A) Effect of LTA on phosphorylation of a protein of *Mr.* 47,000 (P47) in human platelets challenged with PDBu. Platelets were preincubated with LTA (0.5 and 1.0 μg/ml) for the indicated time (10 min) before challenge with PDBu (30 and 50 nM). After addition of PDBu, the reaction mixture was incubated at 37℃ for 1 min with stirring. Lane 1, platelets with Tyrode's solution; lane 2, platelets with PDBu (30 nM); lane 3, platelets with LTA (1.0 μg/ml) and line 4, with LTA (0.5 μg/ml) for 10 min followed by addition of PDBu (30 nM); lane 5, platelets with PDBu (50 nM); lane 6, platelets with LTA (1.0 μg/ml) for 10 min; lane 7, platelets with LTA (2.0 μg/ml) for 60 min. The arrow indicates a protein of *Mr.* 47,000 (P47). (B) The relative detection densities of the radioactive bands were expressed by FSL/mm^2 (FSL, photostimulated luminescence). The data are representative examples of four similar experiments.