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大腸桿菌釋放性內毒素對細菌感染防禦能力的影響

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摘　要

大腸桿菌釋放性內毒素具促進小白鼠對病原性細菌感染之優越防禦能力及長時間的持續作用，其防禦作用受毒素投與途徑及次數之影響。釋放性內毒素會促進巨噬細胞及多形核白血球對 Candida albicans 之吞噬作用及腹腔細胞數明顯的增加。若依白血球及血小板數值，血液 BUN，uric acid 及 creatinine 量及血液 aspartate aminotransferase 及 alanine aminotransferase 活性及臟器 TBA-reactive substances 等加以評估，釋放性內毒素明顯減緩大腸桿菌感染時對肝腎功能之破壞，及臟器之 lipid peroxidation。故釋放性內毒素除活化宿主之非特異性細胞免疫能力直接撲滅細菌外，亦具促進宿主之生理機能，二者共同抵禦細菌之感染及其毒素之解毒作用而達到細菌感染之撲滅。

釋放性內毒素之作用類似結構性內毒素但效果較佳。而腸毒素雖對肝腎功能具保護作用，但對細菌感染之防禦能力則不甚明顯。

革蘭氏陰性細菌其釋放之釋放性內毒素(free endotoxin)自 Cruthley 氏等於 1967 年首次提出後頗受關注[1,2]。該毒素係來自生長中微生物所釋放之脂多醣體而非菌體之分解產物[3,4]。釋放性內毒素是革蘭氏陰性細菌所具有之重要病原性物質及毒性物質[5-9]。本實驗使用之釋放性內毒素和購自 Difco 之結構性內毒素(cellular endotoxin)雖然氨基酸含量略有差異，但兩者之生物活性則頗爲類似[10]。

腸道微生物族群和宿主之關係最近漸受注意，腸道正常菌叢之消長常受外來環境因素之影響，如氣候差異，食物種類，緊張，藥物之投與及外來菌之侵入等，因而影響宿主之生理機能，老化，癌之發生，免疫機能，對細菌感染及各種疾病之產生等，甚致影響到藥物之藥效及營養素之合成及吸收[11]。除厭氧性細菌外，大腸桿菌是腸道之主要族群及維繫其正常功能之微生物。尤其在腸道所產生之釋放性或結構性內毒素及腸毒素對腸道微生物之消長及宿主生理機能之影響頗爲關注[10,12]。故本文擬就釋放性內毒素對活化宿主非特異性細胞免疫能力及對生理機能之保護等結果提出報告。並和結構性內毒素及腸毒素之作用加以比較。

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材料及方法

實驗菌種及材料：試驗菌種係來自臨床糞便之大腸桿菌莢膜多醣類菌株(CPS-4)，該菌除供感染實驗外，亦供釋敎性內毒素及腸毒素之製備[4,9,10]。其他使用之病原性細菌包括 Pseudomonas aeruginosa ATCC 27853，Klebsiella pneumoniae ATCC 13883，Serratia marcescens ATCC 8100，Proteus vulgaris ATCC 13315，Enterobacter cloacae ATCC 23355，及 Staphylococcus aureus ATCC 25923 等株。釋放性內毒素及腸毒素之製備及其特性如前報所述[10]。釋放性內毒素對 NIH 株小鼠腹腔注射及 Wistar 株大白鼠靜脉注射之 LD_{50} 分別是 0.26 mg/kg 及 1.6 mg/kg。結構性內毒素係購自 Difco 的 E.coli 0127：B 8 株其 lipopolysaccharide 係依 Westphal 方法粹取者[13]。

動物實驗：Female ICR 株小白鼠及 Wistar 株大白鼠分別購自台大動物中心，置於溫度調節動物室給與台糖飼料及蒸餾水自由給與一星期後供實驗用，小白鼠及大白鼠之體重分別是 20-25 g 及 250-300 g 之間。小白鼠分別以 4 μg/mouse 釋放性內毒素，結構性內毒素及腸毒素連續腹腔投與 4 天，供巨噬細胞及多形核白血球對 Candida albicans 吞噬能力，大腸桿菌(2 mg/mouse)感染後腹腔細胞數及細菌數值，及其他病原性細菌感染防禦能力等之測定。大白鼠經 10 μg/rat 連續腹腔注射 4 天後，以 4 mg/rat 大腸桿菌腹腔感染之，4 小時後以 sodium pentobarbital 麻醉採取股動脉血供血液學及血清生化學檢查。

巨噬細胞之分離及噬菌能力之測定：每隻小白鼠以 0.5 ml 的 5%glycogen 腹腔注射後 4 天，小白鼠經脫頸致死後，取 5 ml 的 RPMI -1640(Gibco：其中添加 10% fetal calf serum，100 μg/ml penicilllin & streptomycin，5 u/ml heparine)注入腹腔經充分混合後，其腹腔洗液投入置有蓋玻片培養皿中，經 37°CO_2incubator 培養形成單層巨噬細胞後重新放入新鮮 RPMI-1640，同時加入 0.1 ml 經 PBS 清洗 3 次之 brain heart infusion broth (BHI；Difco)培養 3 天之 Candida albicans，再經培養 2 小時後以 PBS 洗除未被吞食之 Candida albicans 後，以 methanol 及 acetone(1：1)混合液固定之，經 Giemsa's 染色後觀察之。

多形核白血球之分離及吞噬能力測定：每隻小白鼠以 0.5 ml 的 5%glycogen 腹腔注射 24 小時後，腹腔注入 5 ml 的 M-199(Difco；其中添加 10% FCS，100 μg/ml penicillin & streptomycin，5 u/ml heparin)，充分混合後，其腹腔清洗液注入培養皿中，經 37°C CO_2 incubator 6 小時後除去附著於玻璃上的細胞。細胞懸浮液經 M-199 洗 3 次(最後細胞數約 1-2×10^7 cells/ml)，然後加入 0.1 ml 經 PBS 清洗 3 次之 BHI broth (Difco)培養 3 天之 Candida albicans，再培養 15 min 後取 0.1 ml 於玻片上，乾燥後以 Leu's 染色之並計算其吞噬率。

血液學檢查包括血容比，白血球及血小板計數。血清生化學檢查包括蛋白質定量[14]，aspartate aminotransferase (GOT; EC 2.6.1.1) alanine aminotransferase (GPT; EC 2.6.1.2)活性[15]，blood urea nitrogen, uric acid, creatinine 之定量[16]及臟器 lipid peroxidation 之測定[17]。

結果分析：全部結果以 mean±SD or SE 或 $\bar{d}\pm SD_{\bar{d}}$ 表示之。以 Student's t-test 做爲實驗組及對照組間統計學意義分析之依據。當 P 值<0.05 則表示具統計學有意義差異。

結　果

投與劑量與防禦能力之影響(如圖 1)：以 2-4 μg/mouse 釋放性內毒素(F-END)及結構性內毒素(C-END)單次腹腔注射後 24 小

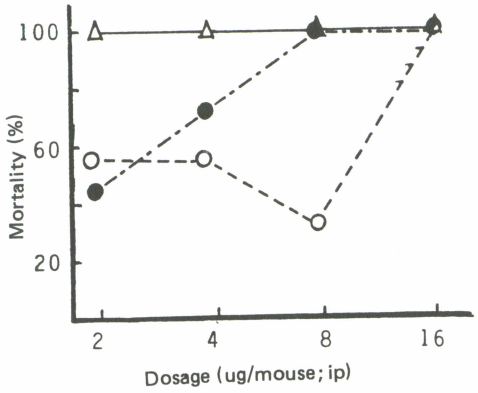

Fig. 1. Protective activity of ICR mice after various doses of free endotoxin (○－－○), enterotoxin(△－△) and cellular endotoxin (●－－●) pretreatment, respectively, the pretreated mice against 2 mg/mouse ip of Escherichia coli infection. The results were observed for a week. Each group consisted of 7 mice.

時，對大腸桿菌(CPS-4；2 mg/mouse)之腹腔感染具防禦作用，當提高到 8 μg/mouse 時 F-END 具較佳之結果但 C-END 則否。若提高到 16 μg/mouse 的 F-END 或 C-END 雖不會致小白鼠死亡，但對 CPS-4 之感染則無保護作用。相反的，由 2-16 μg/mouse 腸毒素(ENT)預處理小白鼠對 CPS-4 株感染都無防禦能力。

注射途徑與抗菌的關係如表 1 所示。以 0.4 mg/kg 的 F-END，C-END 及 ENT 經靜脉或皮下注射 24 小時後，對 2 mg/mouse CPS-4 株感染無防禦效果，除 ENT 外腹腔注射則呈現保護作用。若先以 cemitidine(1mg/kg)腹腔注射 2 小時後再經投與 1.0 mg/kg 的毒素，則都具相當好的抗菌作用，其理由除毒性外不甚明瞭。

注射次數與抗菌的關係(如表 2)：4 μg/mouse 的 F-END 腹腔連續投與 4 次以上對 CPS-4 株之感染可獲良好的防禦效果，但 C-END 則須連續投與 8 次以上。而 ENT 雖多

Table 1. Protective Activity of Free Endotoxin Pretreated Mice Against *Escherichia coli* Infection

Immunizing agent	Mortality(%) Route of administration (Dosage;mg/kg)				
	IV(0.4)	SC(0.4)	IP (0.2)	(0.4)	po(1.0)
F-END	100.0	100.0	57.1	71.4	28.6 *
C-END	100.0	100.0	71.4	85.7	42.9
ENT	100.0	100.0	100.0	100.0	57.1
Control	100.0	100.0	100.0	100.0	100.0

24 hrs after the final pretreatment the pretreated mice against 2 mg/mouse ip *Escherichia coli* infection. the results were observed for a week.

Each group consisted of 7 mice.

* Fisher's exact test: $P<0.05$.

Abbreviation: Free-endotoxin (F-END), cellular-endotoxin (C-END) and enterotoxin (ENT). Intravascular (IV), subcutaneous (SC), intraperitoneal (IP) and per os (PO) administrations.

Table 2. Protective Activity of Free-Endotoxin Pretreated Mice against *Escherichia coli* Infection and Relation to Times of Treatment

Treated with	Mortality (%) Times of treatment (4 μg/mouse/times; i.p.)		
	4	8	14
F-END	0.0*	0.0*	0.0*
C-END	42.9	0.0*	0.0*
ENT	42.9	85.7	42.9.0
Control	100.0	85.7	100.0

Metnology and abbreaviation see Table 1 foot note.
Each group consisted of 7 mice.
* Fisher's exact test: $p<0.05$.

Table 3. Effect of In Vitro Candida albicans Phagocytosis of Intraperitoneal Leukocytes in Free-Endotoxin pretreated mice

Pretreated with (4 μg/mouse/day: 4 days)	Candida albicans phagocytosis (%) (Mean±SE)	
	Macrophage	PMN
F-END	40.2±0.8**	57.5±6.7***
C-END	31.7±4.0**	40.0±11.2*
ENT	32.5±5.4**	57.0±10.8***
Control	20.5±4.9	27.5±2.2

Siginificantly different from cnotrol group (df=8): *<0.05, ** p<0.01 and ***p<0.001.
Ababreviation see Table 1 foot note.

Table 4. Effect of Leukocyte and Extracellular Bacteria Counts of Free-Endotoxin Pretreated Mice Against *Escherichia Coli* Infection

Treated with (4 μg/mouse/day; 4 days)	$\bar{d}\pm SE\bar{d}$ Tail's blood count (mm^3)	Differential count (%); PMN	Extracellular bacteria count ($\times 10^5$ CFU/mouse)	Intraperitoneal leukocytes ($\times 10^6$ cells/mouse)
F-END	+1,220±968	+36.5±2.6*	93±48#	8.55±0.87**
C-END	+1,760±973	+28.0±2.5	513±445	5.70±1.28**
ENT	−2,160±459	+22.2±5.0	1,063±393	5.95±1.49**
Control	+160±542	+24.0±6.7	1,763±275	2.20±0.03

Significantly different from control group (df=8): *P<0.01 and **P<0.001.
Mean±SE. Abbreviation see Table 1 foot note.

次投與其防禦效果不甚明顯。

對 Candida albicans 之吞噬作用如表 3 所示。處理組小白鼠之腹腔巨噬細胞及多形核白血球之吞噬能力較對照組明顯增加($p<0.05$-$p<0.001$)。尤其 F-END 組之巨噬細胞之活性更爲明顯。

釋放性內毒素預處理小白鼠遭受 CPS-4 株感染 4 小時後，其尾部血液白血球數，腹腔細胞數及細胞外細菌數之結果如表 4 所示。以 2 mg/mouse 之 CPS-4 株感染小白鼠後觀察其尾部血液白血球數時發現 F-END 及 C-END 會促使白血球數明顯增加[22]，尤其 F-END 之多形核白血球的增加更爲明顯，相反的，ENT 則減少。由腹腔細胞外細菌的減少及腹腔細胞數的增加，F-END 及 C-END 預處理小白鼠較對照組具明顯的結果，ENT 組之腹腔細胞數雖較對照組明顯增加，但其細胞外菌數則類似對照組。F-END 連續投與 4 次之

小白鼠對 CPS-4 株感染之防禦能力直到第 28 天仍具很好的防禦作用，此種作用持續到第 42 天以後才慢慢消失(如圖 3)。

釋放性內毒素預處理小白鼠對其他病原性細菌感染之防禦能力如圖 4 所示。取 0.5 ml 添加 1% bacterial mucin (Difco)的 18 小時 heart infusion broth 培養菌腹腔注射後觀察其死亡率，由結果顯示 F-END 除對 CPS-4 株感染具很好防禦作用外，對其他病原性細菌如 Staphylococcus aureus, Serratia marcescens, Enterobacter cloacae, Proteus vulgaris, Klebsiella penumoniae 都呈現很好之交叉保護作用，C-END 組除 Enterobacter cloacae 及 Pseudomonas aeruginosa 外其防禦能力較 F-END 組差但仍優於 ENT 組。

釋放性內毒素預處理大白鼠遭受 CPS-4 株感染 4 小時後，其血容比，白血球及血小板數值如表 5 所示，血容比除 C-END 組外類似感染對照組，但 F-END 及 ENT 組之白血球及血小板數值其減少程度則呈現明顯的緩和，相近於未感染對照組。F-END，C-END 及 ENT 組之腎功能指標血清 BUN，uric acid 及 creatinine 量較感染對照組低(如表 6)。三組之血清 GOT 及 GPT 活性其減少更爲明顯。故釋放性內毒素可減少大腸桿菌感染時對肝腎功能之破壞。大腸桿菌感染後對臟器 lipid peroxidation 之影響如表 7 所示。實驗組之 TBA-reactive substances 除 F-END 之腎及 C-END 之肝外，都較感染對照組之數值明顯的減少。

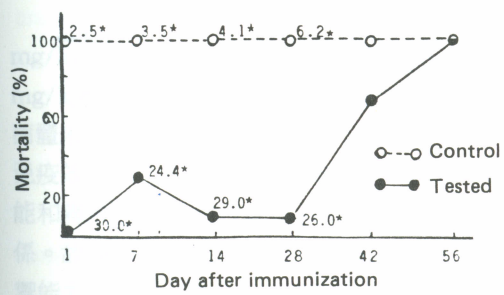

Fig. 2. Protective effect of free endotoxin-immunized mice against 2 mg/mouse of Escherichia coli infection (ip). Each group consisted of 10 ICR mice. * Figure show the LD_{50} (mg/kg).

討　論

非特異性免疫能力對革蘭氏陰性細菌之醫院媒介性或伺機性感染之防禦頗爲重要[11]。腸內細菌共同抗原(Enterobacterial common antigen)曾被做爲腸內細菌屬之共同決定因子[18]，其他如 Anaerobic corynforms 之增強宿主對移植癌細胞[19]，原蟲[20]，細菌[21]及病毒[22]感染之防禦力，Pseudomonas aeroginosa 及 Propionibacterium acnes 及其培養上清液對細菌感染防禦能力之促進等[23-26]。由本實驗之結果，大腸桿菌之釋放性內毒素具促進腹腔巨噬細胞及多形核白血球對 Candida albicans 吞噬能力之有意義增加，及較對照組明顯增加之腹腔細胞數。此種非特異性之表現，使其對病原性細菌之感染如 S. marceceus，E. coli，K. pneumoniae, P. vulgaris, P. aeroginosa 及 S. aureus 等呈現良好之防禦效果。

根據以往之報告[12]，分別以釋放性內毒素及 CPS 株菌體免疫之小白鼠對 CPS 株及其釋放性內毒素之感染或攻擊都具良好之防禦效果。但當菌體先以 mitomycin-C, spectinomycin 及 polymyxin-B 等抗生素處理後則其對小白鼠之致死毒性較未處理者明顯的減弱。而且其免疫小白鼠對釋放性內毒素(0.5 mg/mouse)攻擊之抵抗能力也有明顯的差異，其死亡率分別是 75.0%，87.2%，及 37.5%較菌體未處理免疫小白鼠之零死亡率有明顯的提高，除 polymyxin-B 組外和未免疫對照組之 100%死亡率相似。但以 CPS-4 株感染時則其防禦能力較未免疫對照組(LD_{50}2.5

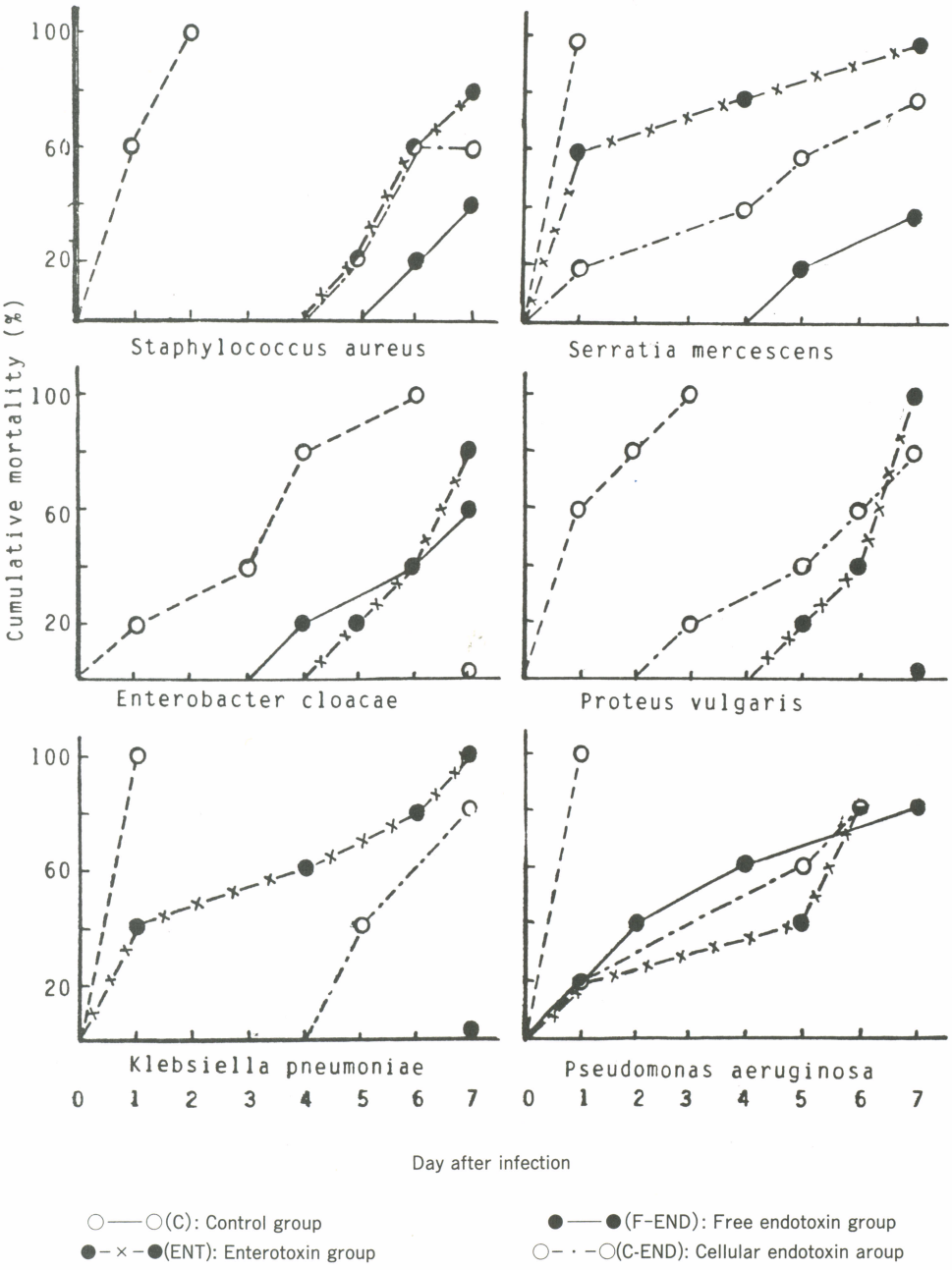

Fig 3. Protective effects of free-or cellular endotoxin and enterotoxin pre-treated mice against heterologous bacteria infection, respectively. Mice were injected intraperitoneally with 4 μg/mouse/times of toxins for 4 times, 24 hr after the final immunogen pretreatment the pretreated mice were respectively challenged intraperitoneally with 0.5 ml/mouse of 18 hr HI broth culture (Bactrol disks, Difco) containing 1% bacterial mucin (Difco) into a mouse of about 25 gm weight (ICR strain), and death was assessed a week later. Each group consisted of 10 mice.

Table 5. Hematological Change Caused by *Escherichia coli* Infection in Free-Endotoxin, Cellular-Endotoxin and Enterotoxin pre-treated Rats

Treated with (10 μg/rat/times)	Hematocrit (%)	WBC (mm^3)	Platelets (mm^3)
F-END*	44.0±1.79	6,740±2.360[b]	381,000±50,800[b]
C-END	40.6±2.65[a]	5,340±2,660	280,000±37,700
ENT	43.2±1.33	5,000±1,900	378,000±62,700[b]
None (Infected)	43.6±5.04	3,140±605	204,000±57,600
Control (Buffered saline)	42.4±1.95	9,880±1,430	343,400±49,300

Each datum is expressed as Mean±SD. Significantly different from None (Infected) group (df=8): [a]$p<0.05$ and [b] $p<0.01$.
Abbreviation see Table I foot note.

Table 6. Effects of Free-Endotoxin, Cellular Endotoxin and Enterotoxin on Renal and Hepatic Functions in Rats Against *Escherichia coli* Infection

	pretreated with (10 μg/rat/times; 4 times)				
	F-END*	C-END	ENT	None (infected)	Control (Buffered saline)
BUN (mg/dl)	25.5±5.4 #	24.1±8.0	32.6±3.2	38.9±10.8[c]	13.0±1.2
Uric acid (mg/dl)	2.80±0.51[a]	2.08±0.04[b]	2.26±0.25[b]	4.50±0.60[c]	3.00±1.50
Creatinine (mg/d l)	0.84±0.17	1.02±0.22	0.84±0.15	1.00±0.14	0.80±0.20
Serum-GOT (μ/ml)	83.5±2.8[b]	88.4±10.7[a]	75.1±11.1[b]	131.1±13.9[d]	79.7±10.6
Serum-GPT (μ/ml)	32.9±1.5[bd]	30.3±3.7[bd]	42.2±8.7[bd]	127.8±14.2[d]	16.8±1.2

Each datun is expressed as mean±SD. Significantly different from None (infected) group (df=8), a $p<0.01$ and b $P<0.001$; and from control (buffered saline) group (df=8), c $p<0.01$ and d $p<0.001$.
*Abbreviation see Table I foot note.

mg/kg)有明顯的提高，其 LD_{50}分別是 23.5 mg/kg，16.9 mg/kg 及 35.9 mg/kg 結果和菌體未處理之免疫組類似(23.5 mg/kg)，此種免疫效果受攻擊源之差異而產生不同之結果可能和釋放性內毒素之存在與否具有密切的關係。故釋放性內毒素免疫小白鼠對細菌感染防禦能力之獲得，除宿主細胞性免疫能力之提高外，可能尚受其他防禦機轉之幫助。

大腸桿菌之釋放性內毒素大量投與時會引起小白鼠生理功能之變化，如血壓，白血球數及血小板數之下降，紅血球數及血容比之增加，及對肝腎功能之嚴重破壞等[10]。Peavy& fairchild 氏等報告，內毒素中毒之小白鼠其肝臟分解酶及脂過氧化酶(Lipid peroxidase)釋放之促進[27]，抑制 NPSH 量及 Superoxidase dismutase 活性[27-29]，內毒素亦會促進血清 total lipid，lipoprotein 及肝 triglyceride 之累積[30]，減少肝 cytochrome p-450 活性及 hepatic microsomal drug 之代謝活性等[31-32]。雖然 Deitch 氏[33]等報告，大腸桿菌 026：136 或 011：134 內毒素微量多次注射，使小白鼠之腸內細菌較易導入腸系淋巴結之報

Table 7. The Effect of Free-Endotoxin, Cellular Endotoxin and Enterotoxin Pre-treatment on Lipid Peroxidation in Rats Against *Escherichia Coli* In-fection

Treated with (10 μg/rat/times; 4 times)	TBA-reactive substances (n mole/mg protein/ml; × 10-4) Heart	Spleen	Kidney	Liver
F-END*	0.597±0.125 #cd	0.780±0.118c	2.518±0.499e	2.302±0.630a
C-END	0.693±0.124b	0.709±0.089a	1.605±0.270cd	3.138±0.482
ENT	0.825±0.086b	0.829±0.321a	1.902±0.515d	2.451±0.184ad
None (infected)	1.483±0.302d	1.364±0.121	2.221±0.221e	3.613±0.767
Control (buffered saline)	0.888±0.152	0.997±0.692	1.141±0.172	3.381±0.703

Each datunis expressed as Mean±SD. Significantly different fron None (infected) group (df= 8), a $p<0.05$, b $P<0.01$ and c $p<0.001$; and from control (buffered saline) group (df= 8), d $p<0.05$ and e $P<0.001$.

* Addreviation see Table I foot note.

告，認爲係因腸粘膜壁障之損傷及 xanthine oxidase 活性之抑制或不活化結果所致，但由內毒素呈現之非特異性細胞免疫能力已足夠克服細菌進入循環系統引起疾病。釋放性內毒素微量投與大白鼠其 hepatic sulfhydryls 及 metalothioneins 量會明顯提高，及 glutathione associated enzyme 活性之促進，導致其對大白鼠之無機或有機汞之慢性或急性中毒時，汞之累積及其毒性明顯的改善[34]且促進對鋅之攝取量[35]，及肝功能等。故釋放性內毒素除具促進宿主非特異性細胞免疫能力直接撲滅細菌之感染外，其對宿主生理機能之促進如肝酵素活性等，增強其對細菌感染時釋放之酵素及內毒素之解毒能力，因而減少宿主標的器官的破壞，共同達到消滅外來細菌之感染。

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Effect of Free-Endotoxin of Escherichia coli on Protective Activity against Bacterial Infection

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ABSTRACT

Intraperitoneal injection of free endotoxin of Escherichia coli in mice could promote protective activity against the pathogenic bacterial lethal infection, and the duration of action sustained long time was noted. The protective action was affected by the injection route and times of free endotoxin administered.

Free endotoxin in vitro distinct enhancement of the phagocytosis of peritoneal macrophage and polymorphonuclear leukocyte against Candida albicans. When bacterial infected, significant increased peritoneal leukocyte number in mice was noted but the number of extracellular bocteria was decreased remarkably.

From a view of the numbers of leukocyte and platelete, the amounts of serum BUN, uric acid and creatinine, the activities of serum GOT and GPT and the lipid peroxidation of organs. We found that free endotoxin could protect hepatic and renal functions against bacteria infection in rats as well as decrease the level of TBA-reactive substances in organs.

Escherichia coli cellular endotoxin was similar in protective activity to that of free endotoxin, but less effective, whereas its enterotoxin exhibited no protective activity against bacterial infection.

Key words: Free endotoxin, Escherichia coli, bacterial infection, Hepatic function.

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