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病原性大腸桿菌之鑑定方法之研究

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摘　要

由大腸桿菌感染小白鼠4小時後，其尾部血液白血球數值及腹腔細胞數之差異變化結果，顯示(1)以病原性較强之菌株感染小白鼠後，其尾部血液白血球數值的變化較少，而非病原性株感染者則有明顯的下降。腹腔細胞數及脾臟細菌數等之變化亦相當一致。(2)尾部白血球數值之變化及腹腔細胞數之增減數值與宿主對病原性株感染的抵抗能力之强弱相一致。即感染後尾部白血球數較感染前明顯減少，而腹腔細胞數明顯增加者，則宿主具較强之抵抗能力，相反則否。

故小白鼠血液白血球計數值及腹腔細胞數值之增減變化，可作爲大腸桿菌病原性株之鑑定及宿主對病原性株抵抗能力之指標。

緒　言

病原性大腸桿菌除本身之定植能力外，亦可產生多種外毒素（腸毒素及小白鼠致死毒素）及內毒素。前曾報告大腸桿菌之病原性與產生之小白鼠致死外毒素息息相關，即外毒素不但是病原性大腸桿菌最重要之病原性因子，也是小白鼠獲得免疫能力之主要抗原物質之一(1)。

病原性大腸桿菌之鑑定，對腸病原性株已有各種生物鑑定法及腸炎血清型鑑定法等，但對腸外感染之病原性株則除以小白鼠致死毒性（LD_{50}）表示外，並無較好的方法(2-6)，若以Proteose peptone No. 3 glycerine salts（PGS）培養基應用於大腸桿菌之分離時，可分爲兩大類，即產生粘稠菌落之莢膜多醣類產生株（Capsular polysaccharide-Synthesizing strain；CPS）及非粘稠菌落株（Non-CPS），以它們對小白鼠之致死能力，人類血清中之繁殖能力、對抗生素之耐性及腸毒素（LT）之產生能力等都具明顯的差異，故莢膜多醣類之產生能力不但可應用於患者腸病原性株之鑑定，亦適合於非腸病原性大腸桿菌之識別(2,7,8)。

但由小白鼠毒性實驗亦發現Non-CPS株部分仍具有病原性，故本文擬探討大腸桿菌感染後小白鼠之週邊血液白血球計數值及腹腔細胞數之變化與病原性的關係。並以其作爲PGS培養基分離後CPS陽性株病原性之再鑑定，且可作爲Non-CPS株病原性衡量之標準，並探討宿主對大腸桿菌感染之抵抗能力。而且以小白鼠週邊血液白血球之計數作爲大腸桿菌病原性鑑定確較傳統LD_{50}等動物試驗方法，不但快速且較簡單又經濟。

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材料及方法

菌種[1,7,8]：大腸桿菌E－4株，得自人類糞便之病原性株，具外毒素（LT腸毒素及小白鼠致死毒素）產生能力，及藥物多劑耐性，在Proteose peptone No.3 glycerine salt（PGS）培養基產生粘稠之菌落（Capsular polysaccharide-synthesizing strain；CPS），其小白鼠（NIH株約20 gm）腹腔注射之LD_{50}是3.15 mg/kg。大腸桿菌E－2株來自糞便之非病原性株（LD_{50}是88.4 mg/kg）對多種藥物均具感受性，爲非莢膜多醣類產生株（Non-CPS）。其它CPS-264及Non-CPS-1株則得自小孩痢疾患者。以上各種菌株平時保存於半固體培養基（Nutrient broth＋0.5％ agar，Difco）中。

試驗動物：Female ICR mice（約20～25 gm），購自台大動物中心，置於本科溫度控制之動物室，以台糖大雞飼料飼養1星期後供爲本實驗使用。

外毒素之製備：依照前報的方法[7,8]，係以40％ Saturated ammonium sulfate沉澱，沉澱物再經Sephadex G-150（Pharmacia Fine Chemicals，Sweden）Column獲得小白鼠致死外毒素。

抗生素之使用：注射用抗生素以sterile normal saline稀釋之，使用之抗生素包括Tetracycline（Lederle；稀釋爲1.0 mg/ml），Gentamicin（Schering；0.2 mg/ml）及Cefazolin（Fujizawa；0.5 mg/ml），本試驗全部使用新鮮製備之抗生素。

LD_{50}之測定：以各種不同劑量已洗過之活菌懸浮液，經腹腔注射於小白鼠後，觀察其於1週內死亡情形，結果以Reed-Muench方法計算之[9]。

尾部血液白血球及腹腔細胞數之計算：以各種濃度的細菌懸浮液（0.5 ml/mouse）注射於ICR株小白鼠，注射前及注射後4小時各作小白鼠尾部週邊血液白血球的計數；而腹腔細胞數之採樣，係將5 ml無菌PBS注入於經注射細菌懸浮液4小時後之小白鼠腹腔內，充分混合後採取腹腔洗液，以白血球計數器計算其細胞數。同時摘取脾臟，並先以滅菌生理食鹽水清洗後，再置於滅菌研磨器加以研磨，然後取各種稀釋液0.1 ml置於液態的Heart-infusion（HI）agar（Difco）中，混合均勻之，經37℃ 48小時培養後計算其菌落。每組以5隻小白鼠之平均值表示之。

抗生素處理後小白鼠對細菌感染之防禦能力：稀釋後之各種抗生素注射小白鼠腹腔經4小時後，再以洗過之細菌懸浮液腹腔感染，4小時後計算其尾部血液白血球數及腹腔細胞數，結果以每組5隻小白鼠之平均值表示之。

結　果

菌株對小白鼠之病原性（如表1）：實驗用之菌株，以其細菌懸浮液對小白鼠之致死能力來觀察，結果以CPS-264之病原性最高，其次是Non-CPS-1及CPS-4而Non-CPS-2株雖然細菌量增加到8.0 mg/mouse ip時仍僅有60％死亡率。其18小時heart-infusion broth之培養上清液對小白鼠之致死毒力仍以CPS-264株最强，其次是CPS-4株，而且都會產生heat-labile enterotoxin，而Non-CPS-1及Non-CPS-2則否。由以上結果得知，雖然病原性强弱有所差異，但CPS-264，Non-CPS-1及CPS-4等株是病原性株，而Non-CPS-2株則屬非病原性株。

週邊血液白血球數之變化：選取3株病原性株大腸桿菌，其中包括CPS株2株（CPS-4，CPS-264）及Non-CPS株1株（Non-CPS-1）及非病原性株1株（Non-CPS-2），分別培養於Heart—infusion broth（Difco）18小時後，以滅菌生理食鹽水洗3次，取2 mg/ml的細菌懸浮液0.5 ml分別注入小白鼠腹腔，4小時後觀察

Table 1. Virulence to Mice of *Escherichia Coli* Cultivated in Heart-infusion Broth

Cell suspension (mg/mouse)	Mortality (No. of death/No. of tested)			
	NON-CPS-2	CPS-4*	NON-CPS-1	CPS-264*
0.018				5/5
0.035				5/5
0.065			0/5	5/5
0.013			2/5	
0.25		0/5	3/5	
0.50		2/5	5/5	
1.00	0/5	4/5	5/5	
2.00	1/5	5/5		
4.00	3/5	5/5		
8.00	3/5			
Supernatant (0.5ml/mouse)	−	+	−	+

A 18 hr heart-infusion broth culture was washed with three times of sterile normal saline, then made into suspensions of different bacterial concentration with sterile normal saline. Each of 5 mice was injected intraperitoneally with 0.5 ml/mouse of washed cell suspension and culture supernatant, respectivelly. The results of mortality were observed for a week.

*Those are heat-labile enterotoxin producing strains, detected by reversed passive latex agglutination.

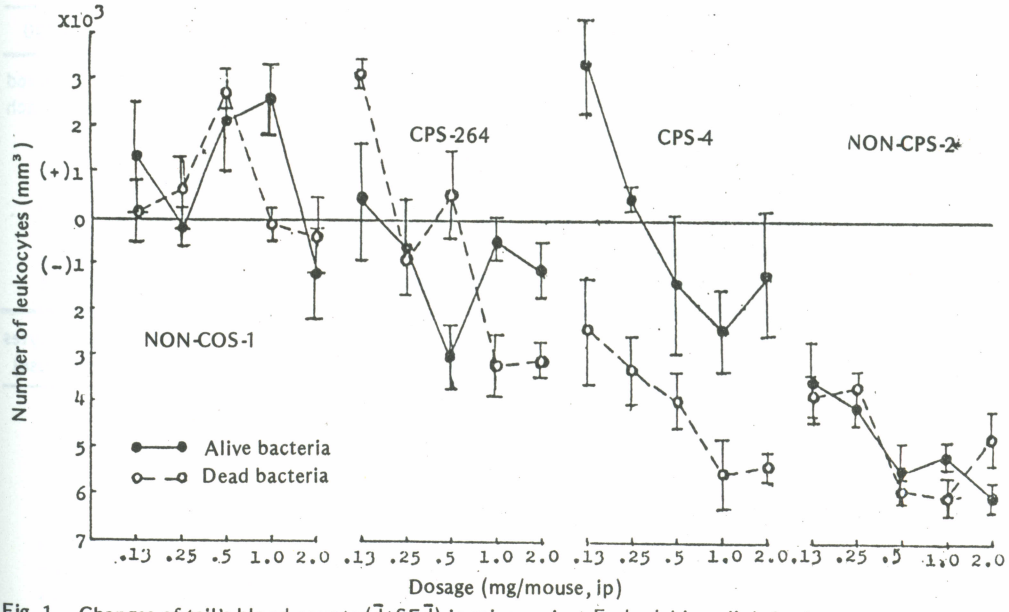

Fig. 1. Changes of tail's blood counts ($\bar{d}\pm SE\bar{d}$) in mice against Escherichia coli infection.
A 18 hr heart-infusion broth culture was washed with sterile normal saline three times, then made into suspensions of different bacterial concentration with sterile normal saline. 0.5ml of each suspension was injected intraperitoneally into a mouse. Each group consisted of 7 mice. The results of tail's WBC count were observed at 4 hr after baterial suspension infection.

其尾部血液白血球之變化，其處理前後白血球之增減如表2所示。病原性株CPS-4，CPS-264及Non-CPS-1等減少1020～1210 cells/mm³，而非病原性株（Non-CPS-2）則減少5000 cells/mm³。可見非病原性株感染小白鼠會引起明顯的動物尾部白血球的減少。

以各種不同濃度之細菌懸浮液注射小白鼠4小時後其變化如Fig.1所示，濃度較小時（0.13～1.0mg/mouse i.p.）病原性株週邊白血球之變化較大，而菌株濃度在2.0mg/mouse時則呈現較爲穩定的變化，白血球的減少率在1000 cells/mm³左右。若以65℃ 30′處理之washed cells suspension感染小白鼠，其中Non-CPS-1株感染時小白鼠白血球之變化與未經熱處理者相似，CPS-264在0.25～0.5mg/mouse之熱處理群則較未處理群之白血球變化少，但1.0～2.0 mg/mouse組則減少較爲明顯。CPS-4株熱處理群則與Non-CPS-2非病原性株之熱處理及未處理群之變化相似，白血球數明顯地下降。由以上結果得知，細菌量以2.0mg/mouse最爲穩定，即病原性株與非病原性株間之變化最爲明顯。Non-CPS-1及CPS-264之熱處理群其白血球數之所以並無減少，甚或增加的現象，是否與細菌之periplasmic space所擁有的其他物質有關，仍待進一步的探討。

細菌懸浮液之清洗是否亦會影響實驗之結果（如表2），若以24小時HI broth培養液腹腔注射，則發現不論是病原性或非病原性株之白血球數都有明顯的下降（4950～9430 cells/mm³）。

週邊白血球之變化與大腸桿菌外毒素的關係（如表3）：小白鼠週邊白血球減少值隨外

Table 2. Changes of Tail's Leukocytes Count of Washed Cells Suspension and HI Broth Culture Infection in Mice

Challenger	No. of leukocytes (mm³) ($\bar{d}$±SE$\bar{d}$)	
	Washed cells suspension (2mg/mouse)	Broth culture (24 hr; 0.5 ml/mouse)
NON-CPS-1	-1,210±1,080	-4,590±1,390
NPS-264	-1,020±630	-4,260±740
CPS-4	-1,050±1,200	-9,430±890
NON-CPS-2	-5,000±812	-6,380±980
Control (HI)	–	- 530±790

The results of the tail's WBC count was observed at 4 hr after bacterial suspension infection. Each group consisted of 5 mice.

Table 3. Changes of Tail's WBC and Total Number of Peritoneal Leukocytes Counts in Mice Against Exotoxin and The Mixture of The Exotoxin and Heat-Inactivated Bacterial Suspension Injection

Exotoxin (mg/kg)	Added with bacterial suspension (2mg/mouse)	Tail's WBC count ($\bar{d}$±SE$\bar{d}$)	Peritoneal leukocytes (x10⁷ cells/mouse)
0.2	None	+2,940±810	1.20±0.20
	Yes	- 590±562	0.80±0.09
2	None	-2,260±795	1.44±0.23
	Yes	+ 730±944	1.02±0.17
20	None	-4,770±665	0.78±0.12
	Yes	-2,320±1,046	0.98±0.14
0	None	-2,360±1,066	–
	Yes	- 500±866	0.40±0.03

Tail's WBC count and peritoneal leukocytes were obtained from the mice 4 hr after intraperitoneal injection with exotoxin and the mixture of exotoxin and heat-inactivated bacterial suspension, respectively. Each group consisted of 5 ICR mice.

毒素量之增加而增加。但若混合熱處理之CPS-4 細菌懸浮液時則變化較不穩定，故熱處理細菌懸浮液會影響外毒素對小白鼠白血球值之計算。總之，外毒素與細菌懸浮液對小白鼠週邊白血球值呈現不同之影響。

抗生素處理後小白鼠對細菌懸浮液感染之影響（如表4）：以 LD_{50} 測定抗生素處理4小時後小白鼠對CPS-4 株感染之抵抗能力，發現抗生素處理群較對照群有明顯的差異，其結果分別是Tetracycline 組增加 7.9 倍，Gentamicin組4.5倍及Cefazolin 組 5.1 倍。抗生素處理群對CPS-4 株感染4小時後其白血球數分別減少 5480 ± 1460 ，5300 ± 1380及 5650 ± 880 cells/mm^3 ，而對照組僅減少 1140 ± 1090 cells/mm^3 。腹腔細胞數值亦較對照組有明顯的增加。由以上結果，證明抗生素確可以增强小白鼠對細菌感染的防禦能力，而此種防禦能力亦可以從週邊血液白血球數值之變化加以鑑定。

小白鼠週邊白血球變化與其防禦能力的關係（如表5）：病原性株感染小白鼠4小時後其尾部血液白血球之減少是 940～1210 cells/mm^3 較對照組之 5200 cells/mm^3 爲少，而且腹腔細胞數 $0.20 \sim 0.37 \times 10^7$ cells/mouse亦較對照組 0.53×10^7cells/mouse 爲少。病原性株感染後小白鼠之腹腔細菌計數值（$> 1000 \times 10^4$ CFU/ spleen ）亦較對照組之 240×10^4 CFU/ spleen 大得多。可見細菌懸浮液感染前後尾部白血球計數值之變化愈少則其腹腔中之PMN數值亦愈少，脾臟之細菌量愈多。此種相關性之變化亦可由 Parental strain及其mucoid mutant 感染小白鼠後實驗結果之變化等加以證實（如表5），故尾部血液白血球計數值變化可作爲大腸桿

Table 4. Changes of Tail's Blood and Intraperitoneal Leukocytes Count of Antibiotics-Pretreated Mice after Bacterial Suspension IP Injection

Pretreated with (μg/mouse)	LD_{50} (mg/kg)	4 hr after 2 mg/mouse of bacterial suspension ip injection	
		Tail's WBC count ($\bar{d}$±SE$\bar{d}$)	Peritoneal leukocytes (x10^6 cells/mouse)
Tetracycline (500)	25.00	-5,480±1,460	0.87±0.25
Gentamicin (100)	14.05	-5,300±1,380	1.51±0.33
Cefazolin (250)	16.20	-5,650±880	1.13±0.17
Control (0)	3.15	-1,140±1,090	0.41±0.06

The results of LD_{50}, tail's WBC count and intraperitoneal leukocyte count were observed for a week and 4 hr after bacterial suspension infection, respectively. Each group consisted of 5 mice.

Table 5. Changes of Tail's WBC Count and Its Relation to the Pathogenicity in Mice

Strain	Tail's WBC count ($\bar{d}$±SE$\bar{d}$)	Peritoneal leukocytes (x10^7 cells/mouse)	Bacteria count (x10^4 CFU/spleen)
CPS-4	-1,050±1,200	0.37±0.15	> 1,000
NON-CPS-1	-1,210±1,680	0.20±0.07	> 1,000
CPS-264	- 940±1,110	0.30±0.08	> 1,000
NON-CPS-2	-5,200±1,200	0.53±0.14	240
Parental strain	-1,360±759	0.39±0.09	> 1,000
Variant (Mucoid)	-3,840±514	0.61±0.12	432

Each group consisted of 5 ICR mice.

菌病原性之參考。

討　論

大腸桿菌是人類腸道主要正常細菌族群，而病原性大腸桿菌不但是引起痢疾之主要病原菌，也是院內感染中主要的革蘭氏陰性桿菌之一。其主要病原性物質包括腸毒素、內毒素及外毒素，而外毒素可能是大腸桿菌菌血症引起繼發性休克之主要成分(1)。

臨床上革蘭氏陰性桿菌的感染率及致死率，有逐漸增加的趨勢，故曾被多數學者所研究。此現象可能有數種因素所促成，宿主抵抗力是克服革蘭氏陰性細菌的最重要因子。而細菌本身之毒性對其致病性也扮演一重要的角色，如某些大腸桿菌K抗原型會引起急性腎盂腎炎，而某些K抗原之存在和大腸桿菌侵入性的增加有關，是大腸桿菌腸道感染很重要的物質(10)。

著者曾報告過大腸桿菌在PGS培養基中具產生莢膜多醣類之能力，對小白鼠之病原性而言，它較血清分型更具密切的關係，莢膜多醣類產生能力與菌株之病原性，產毒性及對抗生素之耐性限閾的提高等具有密切的關係，故莢膜多醣類產生能力可作爲病原性大腸桿菌之分離及鑑定的參考，但此種鑑定方法對Non-CPS株病原性之判斷仍有缺失(2,7,8,11,12,13)。

由本實驗之結果顯示，病原性株在適當條件下對小白鼠感染前後之尾部血液白血球計數值變化較少，非病原性株則呈現頗明顯的白血球減少；而細菌感染小白鼠腹腔後其腹腔細胞數的變化，病原性株較非病原性者明顯減少，脾臟之細菌數則呈現相反的結果，即病原性株感染小白鼠之脾臟充滿了感染細菌。故以尾部血液白血球之計數可對病原性大腸桿菌作鑑定且較傳統方法，即小白鼠之致死毒性（LD_{50}）(3-6)，更簡單可行。

抗生素是傳染性疾患最重要的化學療法之一，以往曾報告(14)抗生素除對感染細菌產生細胞毒性（殺菌及抑菌）外，尚可促進宿主對抗細菌感染之能力，故本實驗擬觀察是否此種作用亦可表現於小白鼠尾部血液白血球數值之變化，結果發現經抗生素處理之小白鼠其細菌感染前後之白血球數值都有明顯的減少，其趨勢與非病原性株感染小白鼠之結果相似；以上結果與病原性株感染之LD_{50}及腹腔細胞數值之增減結果相一致。故小白鼠尾部週邊血液白血球之計數，不但可作爲大腸桿菌病原性之快速鑑定方法，亦可作爲宿主對大腸桿菌感染防禦能力的指標之一。

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Study on An Available Method for The Determination of Pathogenic Escherichia Coli

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SUMMARY

Up to day the identification of pathogenic ***Escherichia coli*** *include some tests in biological activities or serotyping for enterotoxigenic strains. But for the extraintestinal pathogenic strain, the* LD_{50} *in mice was common used. So we planed to observe the variation of WBC counts in mice tail's blood and peritoneal cells. According to these observations, we would discuss the pathogenesis of* ***E. coli*** *and the resistance of host against bacterial infections. We wanted to find a quick and simple identifying method.*

From the results, we could find: 1. After the mice had been infected with ***E. coli*** *the tail's blood WBC counts of nonpathogenic strain decreased clearly, but the change was less in stronger pathogenic groups. The number of peritoneal leubocytes was increased; wherease the bacteria number in spleen was decreased in nonpathogenic strains infection, the opposed results were seemed in pathogenic strains. 2. The change of mice tail's blood WBC counts and peritoneal cells could represent the resistant ability of the hosts against the infections of pathogenic strains. That is the WBC counts decreased clearly after infection but the peritoneal cells increased which means that the host had the stronger resistance. Vice-versa is not true.*

So the change of tail's blood WBC counts and peritoneal cells in mice could to be a indicator for the identification of pathogenic ***E. coli*** *and the resistant ability of host against the pathogenic strains.*

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