行政院國家科學委員會專題研究計劃報告

以 GM6001(Ilomastat)治療廣東住血線蟲感染所引發腦膜炎的效果研究

The efficacy of the therapy with GM6001 in parasitic meningitis caused by *Angiostrongylus cantonensis*-infected mice

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壹、中文摘要

間質蛋白分解酵素-9 被認為參與廣東住血線蟲感染造成的腦膜炎。雖然目前已有一些驅蟲藥可用於治療廣東住血線蟲感染的患者，但無法抑制殘留在腦部的死亡蟲體引起的免疫反應造成腦部的損傷。GM6001 是一種間質蛋白分解酵素專一的抑制劑，其可螯合間質蛋白分解酵素作用時所必需的鋅離子，以阻礙酵素活性，之前的研究已知 GM6001 可阻斷白血球移行及抑制自體免疫疾患造成的腦膜腦炎。本研究的目標是評估 albendazole 配合 GM6001 混合治療廣東住血線蟲感染造成的腦膜炎之效果。Zymography 的分析結果顯示 GM6001 可以抑制 MMP-9 的活性，經過混合治療後的小鼠腦脊髓液及腦組織的 MMP-9 與感染組相比活性明顯減少。感染廣東住血線蟲的小鼠發炎細胞明顯增加，經治療後則明顯減少。混合治療的蟲回收數明顯地較感染組較少。這些結果顯示廣東住血線蟲的感染，以 MMP inhibitor 治療寄生蟲引起腦膜炎是相當好的策略。而 albendazole 配合 GM6001 的混合治療不但可以殺蟲，並可以減少腦部的發炎反應。我們的結論是以 MMP inhibitor GM6001 治療寄生蟲引起的腦膜炎有相當好的效益。

關鍵詞：廣東住血線蟲; 腦膜炎; 間質蛋白分解酵素; GM6001; Albendazole

Abstract：

Matrix metalloproteinase 9 (MMP-9) is involved in the *Angiostrongylus cantonensis* infected meningitis. Recently, some effective anthelmintics have been proposed to treat the *A. cantonensis* in humans. But worm dead bodies leaving in the brain still evoke severe immune response resulting in brain damage. The MMP-specific inhibitor GM6001 acts by chelating the zinc cation in the active site of MMPs, in previous has been reported to block leukocyte migration and suppress autoimmune encephalomyelitis. In an attempt to prevent brain damage caused by worm migration and MMP-9 destruction during *A. cantonensis* infection, we were combine anthelmintic albendazole and MMP inhibitor GM6001 to kill worms and block MMP-9 activity, respectively. The aim of the present study was to estimate the efficacy of this combining therapy in parasitic meningitis caused by

A. cantonensis infection. In a zymography assay GM6001 was able to inhibit MMP-9 activity, the activity in cerebrospinal fluid (CSF) and brain tissues were significantly decreased in combing treatment compared to infected mice with parasitic meningitis. Inflammatory cells were significant increased in infected-mice, and decreased the numbers after treatment. Recovered larvae in combing treatment were significantly decreased, compared to the infected mice. These data suggest that inhibition of MMP-9 may be an effective approach to prevent brain inflammatory reaction caused by *A. cantonensis* infection. Combing therapy with albendazole and GM6001 seem to be better agents to kill the worm and prevent brain damage. We conclude that an MMP inhibitor have a significant beneficial effect in parasitic meningitis.

Keywords: *Angiostrongylus cantonensis*; meningitis; matrix metalloproteinase 9; GM6001; albendazole.

貳、緣由與目的

廣東住血線蟲(*Angiostrongylus cantonensis*)俗稱鼠肺蟲,早在 1935 年由陳心陶先生於廣東省的家鼠(*Rattus norvegius* and *R. rattus*)中發現,其幼蟲寄生於腦中,發育成熟後移行至肺動脈中。在太平洋及東南亞地區,大部份罹患嗜伊紅性腦膜炎的患者是因此寄生蟲寄生於腦部所引發,其感染途徑是因人攝食了感染廣東住血線蟲第三期幼蟲的蝸牛所造成,此幼蟲會穿過消化道移行至腦、脊髓、及眼部而引起疾病,。在台灣,即使公共衛生教育已很普及,但居住在鄉村及山地部落的居民仍常食用遭受廣東住血線蟲感染的蝸牛,使得台灣每年仍有數例感染廣東住血線蟲的病例發生; 目前已有一些驅蟲藥可用於治療遭受廣東住血線蟲感染的患者,但殘留在腦部的死亡蟲體仍會引起嚴重的免疫反應造成腦部的損傷。

廣東住血線蟲造成的腦膜炎致病機轉尚不十分清楚,蟲體移行造成的機械傷害及嗜伊紅性白血球所釋放的神經毒素皆被認為與其致病機轉有關,但詳細機制仍不清楚。在我們之前的實驗中發現於廣東住血線蟲感染的鼠腦組織中出現大量的間質蛋白分解酵素 9 (MMP-9),此酵素在正常鼠腦中並不出現,且此酵素主要位於血管的基底膜以及浸潤於腦中的巨噬細胞。而目前已知在組織發炎及修復的過程中,間質蛋白分解酵素扮演極重要的角色,包括分解血管基底膜及間質結締組織, 而近年來也發現在許多神經退化性疾病, 如多發性硬化症(multiple sclerosis)及阿滋海默症(Alzheimer's disease)的致病機轉中,間質蛋白分解酵素也扮演極重要的角色,與神經細胞的死亡有關。因此我們認為間質蛋白分解酵素 9 可參與廣東住血線蟲感染造成的腦損傷。

GM6001 是一種廣效性的間質蛋白分解酵素抑制劑,其可螯合住間質蛋白分解酵素作用時所必需的鋅離子,以阻礙酵素活性,之前便已知 GM6001 可阻斷白血球移行,抑制自體免疫疾患造成的腦膜腦炎,防止因顱內出血引起的腦水腫,以及防止細菌內毒素引起的死亡。在廣東住血線蟲感染過程中,為了防止蟲體移行及間質蛋白分解酵素 9 所造成的腦部損傷,我們計畫同時投與驅蟲藥 Albendazole 來殺死蟲體及間質蛋白分解酵素抑制劑 GM6001 來抑制間質蛋白分解酵素 9,來治療廣東住血線蟲感染症,本研究的主要目的是要瞭解間質蛋白分解酵素 9 在廣東住血線蟲感染造成的腦部損傷中所扮演的角色,同時評估這種同時給予驅蟲藥及間質蛋白分解酵素抑制劑的結合療法之療效為何。

參、材料與方法

一. 廣東住血線蟲第三期幼蟲(L3)之收集:

由田野撿拾非洲大蝸牛。

↓將非洲大蝸牛外殼碾碎，取其組織，剁碎。

↓用組織均質器絞碎。

↓以 1：30 比例加入人工胃蛋白酵素消化液。

↓以磁性攪拌子於 37℃之恒溫箱中，均勻攪拌消化 2 小時。

↓以雙層紗布濾去雜質，加入生理時鹽水稀釋並靜置。

↓每隔 30 分鐘到去約一半的上清液。

↓再加入生理時鹽水稀釋靜置，重複至完全清澈為止。

↓以滴管吸取下層之沈澱物，置於玻璃皿中。

↓置於解剖顯微鏡下，觀察並吸取 L3 幼蟲。

↓每 30 隻 L3 幼蟲為一單位，置於玻璃皿中。

二、實驗動物

ICR(Institute for Cancer Research)品系小鼠(Mice)，購自國科會動物中心，為三週齡雄性小鼠。感染前至少飼養於動物房一週，並維持在 12 小時亮及 12 小時暗的動物飼養中心。

三、動物感染

小白鼠(ICR strain)在感染前 12 小時均給予禁水、禁食，每隻小白鼠以口胃管分別灌入 60 隻第三期幼蟲(L3)，於感染後 12 小時再恢復其供水、供食。

四、GM6001 的投與

將感染的動物隨機分四組,兩組對照組,兩組實驗組,實驗組分別在感染後 5 天或 10 天,以腹腔注射 GM6001,劑量為每公斤體重 100 毫克,每天注射一次,共注射 7 天,之後禁藥一天,隔日將動物犧牲,而對照組則注射生理食鹽水,在犧牲實驗組動物時也同時犧牲對照組動物,作比較。

五、Albendazole 及 GM6001 的投與

將感染的動物隨機分四組,兩組對照組,兩組實驗組,實驗組分別在感染後 5 天或 10 天,以腹腔注射 GM6001,劑量為每公斤體重 100 毫克,每天注射一次,同時以口胃管投與 Albendazole,劑量為每公斤體重 25 毫克,共投與 7 天,之後禁藥一天,隔日將動物犧牲,而對照組則注射及管灌生理食鹽水,在犧牲實驗組動物時也同時犧牲對照組動物,作比較。

六、病理檢查

動物犧牲時,收集全血,分析血中嗜伊紅性白血球數目,接著將腦蓋剝開,評估其腦膜出血情形,之後取出腦置於培養皿中,將腦膜剝離,以生理食鹽水沖洗,收集此液作為腦脊髓液,分析其中伊紅性白血球數目及 MMP-9,接著以解剖顯微鏡將蟲體挑出,計算其數目,最後將腦固定,作切片,以 H-E stain 染色後觀察。

七、分析 MMP-9 的方法

1、膠體的配製法(Gel Preparation)

同 SDS-PAGE，不同的是 Separating Gel 加入 0.1% 的 Gelatin。

2、0.1%Gelatin SDS-PAGE 電泳後 gel 之處理：

↓取下 gel, 加入 100ml Washing buffer(2.5% Triton X-100 in dH_2O) washing gel。

↓在室溫下搖動 30 分鐘，換 Washing buffer 再洗一次。

↓倒掉 Washing buffer，gel 以 dH_2O washing。

↓加入 200ml Reaction buffer (40 mM Tris-HCL,pH 8.0, 10 mM $CaCl_2$, 0.01%NaN_3)。

↓Incubation at 37℃, 搖動 12 小時以上。

↓Stain gel with Staining solution (0.25% Coomassie Blue R250,

in 50%MeOH, 10%Acetic acid), for 1 hr。

↓ Destain gel with Destaining solution (20%MeOH, 10% Acetic acid)。

肆、結果

Zymography 結果顯示廣東住血線蟲感染的 CSF 及腦組織之 MMP-9 表現量明顯增加，經 albendazole 及 GM6001 治療的腦組織及 CSF 之 MMP-9 表現明顯減少，尤其 Albendazole 及 GM6001 混合治療的腦組織及 CSF 之 MMP-9 表現明顯更少。將 bands 量化的結果顯示治療前與治療後有顯著的差異，顯示 GM6001 治療後確實可以減少 MMP-9 的表現(圖一)。血液分析結果顯示廣東住血線蟲感染的 CSF 中之 neutrophil、macrophage 及 eosinophil 明顯增加，經 albendazole、GM6001 及 albendazole 與 GM6001 混合治療後則明顯減少(圖二)。此結果顯示經治療後可減少發炎細胞的數目。組織切片 Hematoxylin-eosin 染色結果顯示，在腦膜下的蜘蛛膜下腔中有大量的發炎細胞浸潤，有 PMN 及 leukocytes, 尤其是 eosinophil, 經過 albendazole, GM6001 及 albendazole 與 GM6001 混合治療後，蜘蛛膜下腔中之 leukocytes (eosinophil)則明顯減少(圖三)。腦部幼蟲回收結果顯示廣東住血線蟲感染及 GM6001 治療的腦組織可回收較多的虫，顯示 GM6001 並無殺蟲效果，而單獨 albendazole 及 albendazole 與 GM6001 混合治療的腦組織幼蟲回收則明顯減少，顯示 albendazole 殺蟲效果相當好(圖四)。

伍、討論

對於廣東住血線蟲感染所引起的腦發炎反應，MMP-9 確實參與寄生蟲引起的腦膜炎。過去的研究報告(Paul et al., 1998; Leib et al., 2000; Noble et al., 2002)顯示 MMP inhibitors 可以明顯減少因細菌引起的腦部傷害及發炎反應，而寄生蟲引起的腦膜炎則仍無報告證實，本研究以 MMP inhibitor (GM6001)治療廣東住血線蟲感染小鼠所引起之腦膜炎，結果顯示可以明顯減少 CSF 中之白血球，使腦膜發炎的症狀減輕。對於廣東住血線蟲感染的病人，蔡等人(2001)的研究建議以 albendazole 及 steroids 混合來治療，然而類固醇雖然可以控制廣東住血線蟲感染造成的腦膜炎及蟲體被殺死後引起的發炎反應，但是類固醇的使用仍然備受爭議。本研究以 GM6001 取代 steroids，以 GM6001 抑制 MMP-9 可以減少發炎細胞的移行與浸潤，並可避免蟲體被殺死後引起的發炎反應。本研究是第一個以 MMP inhibitor 治療寄生蟲引起腦膜炎而有相當效益的報告，本研究的結果顯示 albendazole 可以殺死蟲體，而 MMPs inhibitor 可以減輕發炎反應，因此以 albendazole 配合 GM6001 治療廣東住血線蟲的感染應是相當好的策略。

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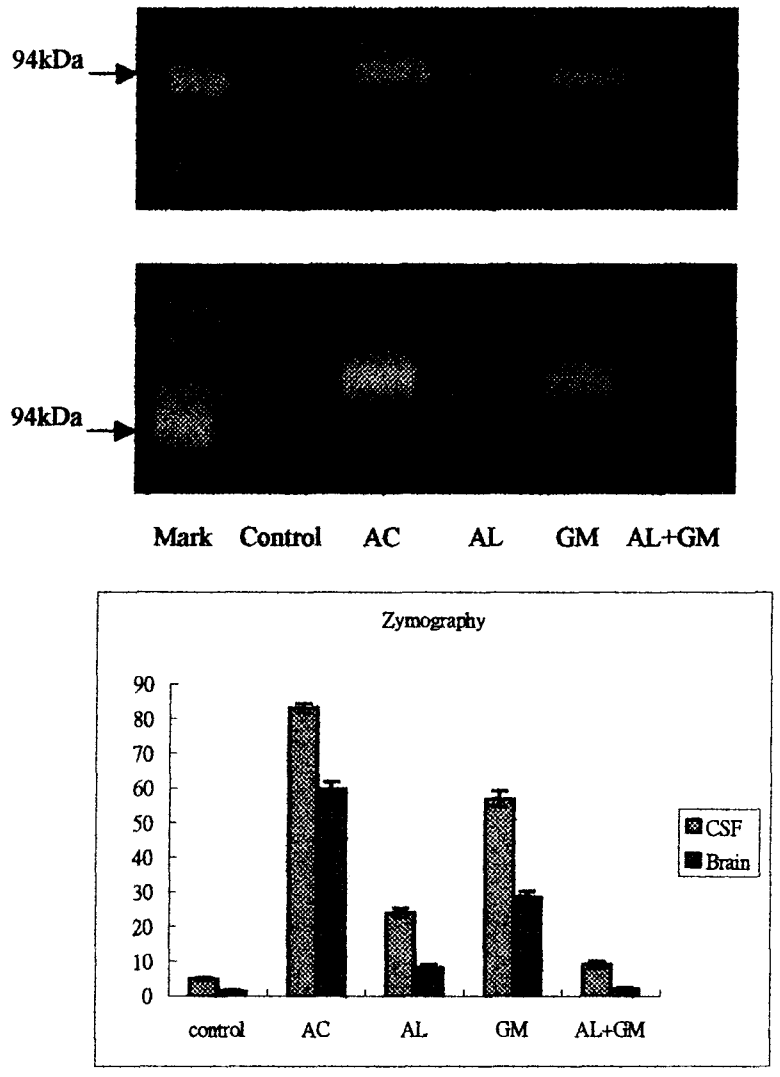

Fig. 1. Detection of MMP-9 activity by gelatin zymography. (A) Zymography of the CSF and brain tissue. Activity of MMP was significant increased in CSF and brain tissues infected-mice; treatment with albendazole or GM6001 decreased the activity of MMP-9; combing treatment with albendazole and GM6001 were significantly decreased the activity of MMP-9. (B) Bands were quantified by densitometry. Open bars represent the density of CSF, solid bars show the density in the presence of brain tissues. The data shown are the mean values and standard deviations (error bars).

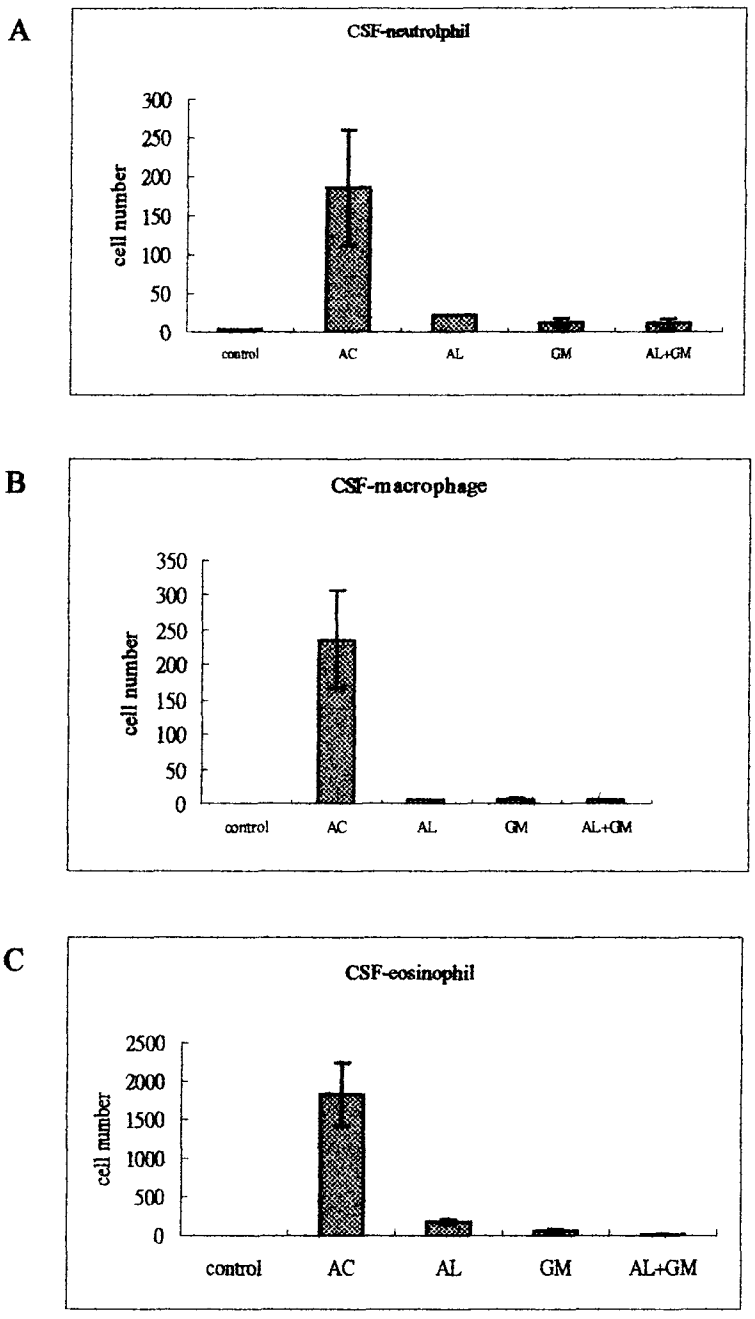

Fig 2. Changes in the leukocyte counts. (A) Neutrophils were significant increase in infected-mice and decreased the numbers after treatment. (B) Macrophages were significant increase in infected-mice and decreased the numbers after treatment. (C) Eosinophils were significant increase in infected-mice and decreased the numbers after treatment. The data shown are the mean values and standard deviations (error bars).

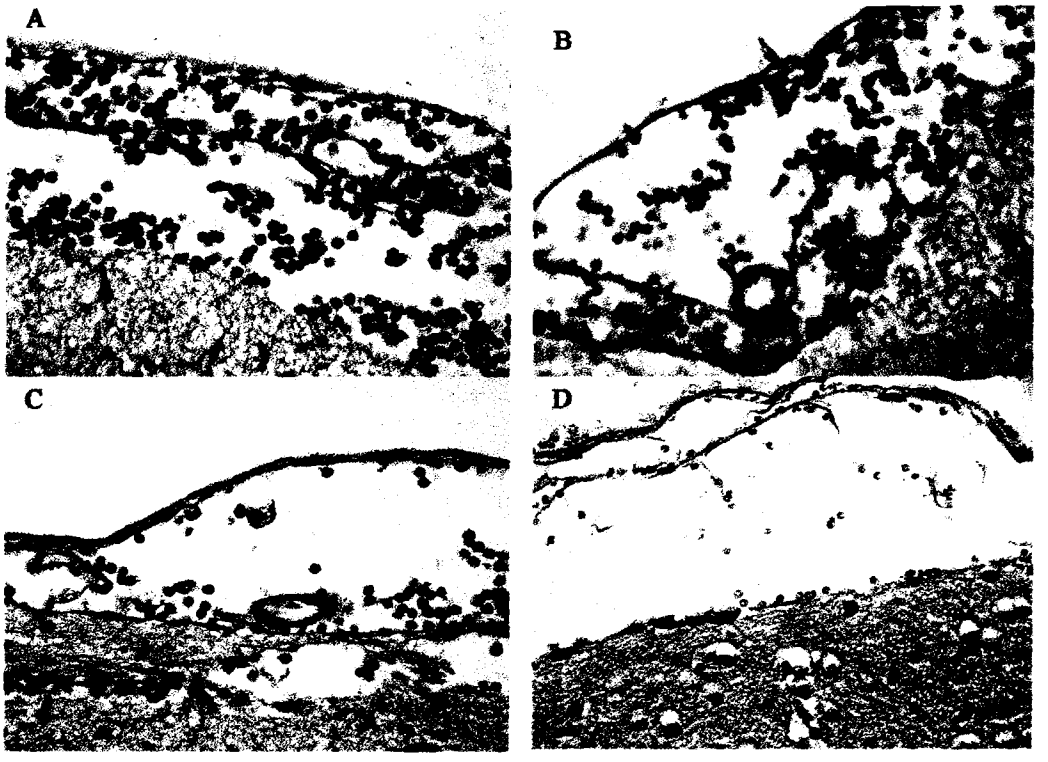

Fig 3. Hematoxylin-eosin staining of inflammatory cells in paraffin sections of the subarachnoid space. (A) The subarachnoid space of infected-mice showing many leukocytes accumulation. (B,C) Treatment with albendazole or GM6001 decrease the leukocyte numbers. (D) Combing treatment with albendazole and GM6001 significantly decreases the leukocyte numbers.

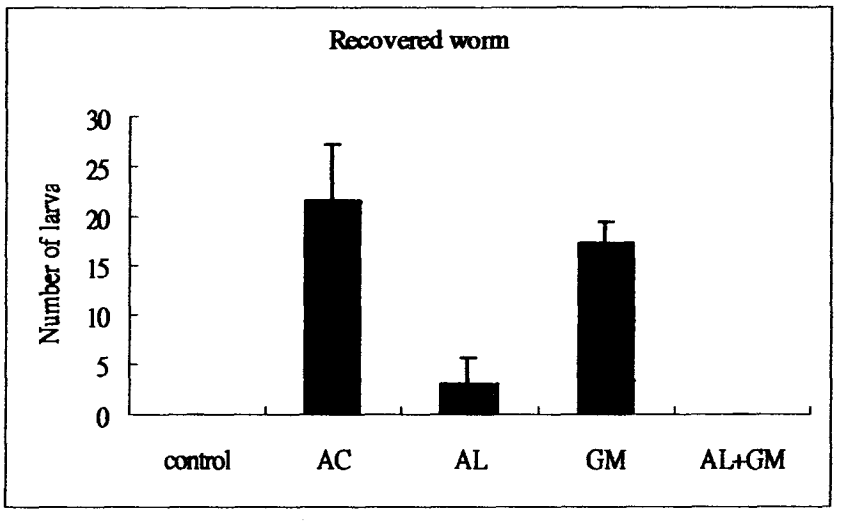

Fig 4. Recovered larvae in GM6001 treated mice were lower than infected mice; treatment with albendazole or combing treatment with albendazole and GM6001 were significantly decreased, compared to the infected mice. The data shown are the mean values and standard deviations (error bars).