東海大學生命科學系博士論文

台灣鋏蠓(小黑蚊)過敏臨床表徵、免疫機轉、
過敏原分子選殖與鑑定之研究

Clinical characterization, immune mechanisms, allergen cloning and identification of *Forcipomyia taiwana* (biting midge) allergy

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誌　　謝

僅以此本論文獻給我的恩師、摯友以及永遠無私地支持我的先生與家人

目　　錄

中文摘要

台灣鋏蠓(俗稱小黑蚊)是一種體型微小(1-1.5 mm)的吸血昆蟲,廣泛分佈於臺灣近郊及鄉間,中國大陸南方也有報告其蹤影。我們發現約60%曾被小黑蚊叮咬的民眾會對小黑蚊叮咬產生過敏反應且小黑蚊叮咬過敏有兩種臨床型態 1)立即型：患者於叮咬 1 小時內產生局部大塊紅腫劇癢反應 2)延遲型：患者於叮咬 12 至 24 小時後生劇癢之丘疹及水泡,甚至皮膚潰爛。此種延遲型皮膚過敏反應往往持續數週甚至數月方消，造成患者極大困擾。我們的研究分為兩大主軸：一為兩種台灣鋏蠓過敏反應的免疫機轉；二為鑑定與選殖台灣鋏蠓主要過敏原。我們發現，小黑蚊叮咬之立即型反應為 IgE 媒介之過敏反應, 且 22-、24-、 35/36- 與 64-kDa 之蛋白質為主要之過敏原。我們的研究也發現免疫球蛋白 E 非導致延遲型過敏反應之主要分子，Th1 免疫反應之丙型干擾素及先天免疫反應之第六介白質與甲型腫瘤壞死因子均參與延遲型過敏反應，顯示 Th1 細胞免疫反應與台灣鋏蠓延遲型過敏反應可能比較有關。蛋白胺活化路徑(protease-activated pathway)可能也參與台灣鋏蠓叮咬過敏之免疫反應。我們用 λZAP II 嗜菌體建立台灣鋏蠓之 cDNA 基因庫，並以病患血清或 For t 2 專一性抗體來篩選菌株。我們將與病患血清 IgE 或

For t 2 專一性抗體反應之選株次選殖至 pET30 或 pQE30 載體並以 *E. coli* 表現系統表現並及純化其重組過敏原蛋白質。我們成功表現並純化一 24kDa 融合重組過敏原(rFor t 1.0101)及一 36kDa 重組過敏原(rFor t 2.0101)。 rFor t 1.0101 與 67.8%之病患 IgE 反應而 rFor t 2.0101 與 75%之病患血清反應, 均符合主要過敏原之定義。rFor t 1.0101 與埃及斑蚊之 serine/threonine protein kinase 有 65.3%之序列相似性而 rFor t 2.0101 序列與埃及斑蚊之 eukaryotic translation initiation factor 3 subunit 有 77.9%之序列相似性。 雖然小黑蚊叮咬過敏往往造成患者生活品質之極大困擾，目前卻沒有標準的檢驗方法可供確立診斷，除了請病患僅量避免被叮之外，也沒有積極有效之治療方式。我們選殖出兩個台灣鋏蠓之主要過敏原, rFor t 1.0101 及 rFor t 2.0101，希望這些純化之台灣鋏蠓重組過敏原，將來可做為檢驗台灣鋏蠓過敏及皮膚試驗試劑之材料及穩定來源進而做為台灣鋏蠓過敏減敏治療疫苗之來源。

英文摘要

Forcipomyia taiwana is a tiny (1-1.5 mm) blood-sucking midge that is distributed island-wide in urban and suburban Taiwan as well as in southern China. Our study aimed to elucidate the clinical characteristics and immune mechanisms of *F. taiwana* allergy in addition to identify and clone the major *F. taiwana* allergens. Our study revealed that about 60% of the subjects develop hypersensitivity reactions to *Forcipomyia taiwana* bites. There are two types of reactions after bites: 1) immediate reaction: large local swelling develops within one hour of bites 2) delayed reaction: intense itching papules and vesicles occur 12 to 24 hours after bites and may last for weeks or even months. We found that the immediate large local reaction is IgE-mediated and the 22-, 24-, 35/36- and 64-kDa proteins are major *F. taiwana* allergens. However, IgE is not the major molecule mediated delayed reactions to *F. taiwana* bites. We found that PBMC from patients with solely delayed reactions markedly proliferated and secreted more interferon-gamma, interleukin-6, and tumor necrosis factor-alpha than the non-allergic subjects suggested that a Th1 type inflammation may be involved in delayed reactions. Protease- activated pathway may also involve in *F. taiwana* allergy. We constructed a ZAPII cDNA library using *F. taiwana* mRNA and then screened by plaque immunoassay with pooled patients' sera or rabbit anti-For t 2 antibody. The cDNA insert of IgE-reactive and anti-For t 2-reactive clones were sequenced and subcloned into the expression vectors, pET30 or pQE30. A 24 kDa recombinant fusion protein (rFor t

1.0101) and a 36kDa (rFor t 2.0101) nonfusion recombinant protein were obtained and purified by His-tag affinity column. The recombinant For t 1.0101 reacted with 67.8% of the patient IgE by ELISA and shows 65.3% sequence homology to serine/threonine protein kinase of *Aedes aegypti* (mosquito). The recombinant For t 2.0101 reacted with 75% of the patient IgE by ELISA and shows 77.9% identity to eukaryotic translation initiation factor 3 subunit of *Aedes aegypti* (mosquito). We cloned two major *F. taiwana* allergens, rFor t 1.0101 and rFor t 2.0101. The recombinant *F. taiwana* allergens will become a useful source for future development of standard diagnostic tool and immunotherapy vaccine for biting midge allergy.

第一章 研究背景及文獻回顧

1.1 過敏反應 (Allergic Reaction)

過敏是個體對無害的外來物質產生過度且不必要的免疫反應而引起組織或器官發炎甚至功能受損的疾病。 早期的文獻多半以 anaphylaxis 一詞描述此一特異之免疫反應[1]，直至 1906 年 Clemens von Pirquet 在維也納一醫院小兒科的猩紅熱病房觀察施打抗毒素血清的病患之特異反應，才首先提出”allergy”一字[2]。Allergy 一字由希臘文 allos (“other”)及 ergon (“work”)所組合而成，來描述原應對病患有治療效果的抗毒素血清反而產生不良反應的現象。

2003 年世界衛生組織將幾個常用來描述臨床異常反應的名詞予以定義：敏感 (hypersensitivity) 是指暴露於正常人可以耐受之刺激物後, 出現客觀可再現之不適症狀，英文也可使用 sensitivity 來形容此一特殊狀況，如阿司匹林敏感型氣喘(aspirin-sensitive asthma)即屬此類。引起敏感的原因，可以是免疫反應所引起，也可以是非免疫反應所引起。過敏(allergy)則是特指由免疫機轉所引起之敏感反應(a hypersensitivity reaction initiated by immunologic mechanisms)[3;4]。

Ishizaka 等人首先於 1960 年代初期發現 IgE 為引起過敏反應的重要媒介[5;6]。傳統上臨床醫師及免疫學者最常使用 Coombs 及 Gell 於

1963 年提出之四種不同的免疫機轉來分類過敏反應[7]。 Coombs 及 Gell 在書中將過敏反應分為 1) 第一型過敏反應：為 IgE 媒介之立即型過敏反應 2)第二型過敏反應：為 IgG 媒介之細胞毒性反應 3)第三型過敏反應：為免疫複合體所媒介 4)第四型過敏反應：延遲型過敏反應，又稱為細胞媒介反應。

隨著免疫學的進步及對過敏反應的了解，Kay 於 1997[8]及 2008[1]於其主編之書中將 Coombs 與 Gell 之分類予以修正，更完整地敘述了過敏反應的樣貌。Kay 提出的 Modified Coombs and Gell 分類將過敏反應進一步細分為七種型態：

1) 第 I 型過敏反應 (Type I)：立即型，IgE 依賴或全身性過敏反應 (IgE-dependent or anaphylactic hypersensitivity)，是過敏原與已被過敏原專一型免疫球蛋白 E (allergen-specific IgE) 致敏之肥大細胞或嗜鹼球交互作用，而引發上述細胞釋放出組織胺等發炎介質所引起。如過敏性鼻炎或過敏性氣喘之急性症狀，藥物或食物引起的全身性過敏反應(anaphylaxis)等。

2) 第 IIa 型過敏反應 (Type IIa)：細胞毒殺反應(cytolytic or cytotoxic reactions)，IgG 抗體與細胞表面之抗原交互作用引起，加上補體系統之作用，造成細胞之崩解死亡。如自體免疫溶血性貧血。

3) 第 IIb 型過敏反應 (Type IIb)：細胞刺激反應(cell-stimulating reactions)，是對細胞有刺激作用之 IgG 抗體與細胞表面之受體作用後，進而影響細胞之訊息傳導與功能。如慢性自體免疫蕁麻疹、自體免疫甲狀腺炎(Graves disease)

4) 第 III 型過敏反應(Type III)：與舊分類同，為 Arthus 型或抗原-抗體免疫複合體媒介。抗原-抗體免疫複合體於活化補體系統後，再引起一連串之發炎反應。如血清病 (serum sickness)、外因性過敏性肺炎(extrinsic allergic alveolitis)等。

5) 第 IV 型 Th1 過敏反應(Type IV Th1)：典型之延遲型過敏反應，此型之過敏反應的特徵，可見到被抗原(過敏原)致敏而活化的 CD4+ 第一型幫助型淋巴細胞 (T helper type 1 cells，Th1 cells) 與巨嗜細胞 (macrophage) 浸潤於病灶處。這些第一型幫助型淋巴細胞的主要特性，為受到抗原刺激後，會分泌丙型干擾素 (interferon-gamma，IFN-γ) 及第二介白質 (interleukin-2，IL-2)。如過敏性接觸性皮膚炎、類風濕性關節炎，結核菌素反應等皆屬此類。

6) 第 IV 型 Th2 過敏反應(Type IV Th2)：CD4+第二型幫助型 T 巴細胞 (T helper type 2 cells，Th2 cells) 被抗原(過敏原)活化致敏後引起。這些第二型幫助型淋巴細胞的主要特性，為受到抗原刺激後，會分泌第四介白質(interleukin-4，IL4)、第五介白質(interleukin-5，

IL-5)、第九介白質(interleukin-9，IL-9)與第十三介白質(interleukin-13，IL-13)。第 IV 型 Th2 過敏反應發炎病灶會有許多嗜伊紅性白血球與嗜鹼性白血球(eosinophil- and basophil-rich inflammatory response)之浸潤。如慢性氣喘、慢性過敏性鼻炎、異位性皮膚炎等。

7) 第 IV 型細胞毒殺過敏反應(Type IV cytotoxic)：組織因毒殺型 T 淋巴細胞之作用而受損。毒殺型 CD8+T 淋巴細胞辨認出標靶細胞表面抗原之片段並進而攻擊之。如早發型胰島素依賴型糖尿病、移植器官之排斥等。

隨著免疫學的進展，雖然過敏反應的分類，由四型逐漸增加到七型，但大部份臨床醫師或病患所稱之過敏病，多半指的仍是第一型之立即型過敏反應。典型的第一型 IgE 媒介之過敏症狀，通常在接觸過敏原數分鐘至數小時內 (很少超過兩小時) 發生氣喘發作、流鼻水、打噴涕、鼻塞、眼睛紅癢、腹痛嘔吐腹瀉、急性蕁麻疹、血管性水腫或過敏性休克。其症狀及嚴重程度端視不同過敏原之免疫特性、進入人體之途徑及過敏原暴露量而有不同。臨床上我們要診斷患者有一 IgE 媒介之過敏反應，除可靠之過敏病史外，醫師還可用過敏原皮膚試驗或檢測血液中過敏原專一型免疫球蛋白 E (allergen-specific IgE) 來證實[9]。

如前所述，隨著近年對過敏機轉的研究，我們知道 IgE 媒介之過敏發炎反應，還有多種免疫細胞參與其中，包括嗜伊紅性白血球 (eosinophils)、 嗜鹼性白血球 (basophils)、肥大細胞 (mast cells) 及 T 與 B 淋巴球等[10]，而且先天免疫細胞及一些非 IgE 媒介之發炎反應，如蛋白腖活化路徑(protease-activated pathway)、類鐸受體(Toll-like receptors)在過敏反應也有極重要的角色[11-14]。

1.2 過敏原之命名

過敏原是指會誘使免疫細胞製造 IgE 或與 IgE 結合(binding)而引起過敏反應的抗原[15]。大部分會與 IgE 反應的過敏原是蛋白質，通常為具有一碳水化合物支鏈之醣蛋白，但很少數的狀況下，純碳水化合物也可以成為一過敏原[16;17]。

根據 1986 年及 1994 年世界衛生組織與國際免疫學會聯盟過敏原命名委員會(World Health Organization and the International Union of Immunological Societies'Allergen Nomenclature Sub-Committee, WHO/IUIS)制定之規則，過敏原的命名是根據其生物學林奈分類系統(Linnean System)的拉丁文學名，以屬名的前三個字母、空一格、加上種名的第一個字母、 空一格、再加上一阿拉伯數字來命名。此阿拉伯數字通常是根據過敏原發現之順序或臨床重要性來排序[18-22]。 如 Lol p 1 為裸麥 *Lolium perenne* 花粉第一個被發現的過敏原，而 Cyn d 1

則為狗牙根草 *Cynodon dactylon* 第一個被發現的過敏原。但在某些過敏原，如家塵蟎，具分子構造上同源型(structural homology)者，會被給予相同之阿拉伯數字。如歐洲蟎 (*Dermatophagoides pteronyssinus*)，美洲蟎 (*Dermatophagoides farinae)*，梅氏嗜黴蟎 (*Euroglyphus maynei*) 及熱帶無爪蟎 (*Blomia tropicalis*) 之第一群過敏原(group 1 allergens)均具有 cystein protease 之特性，分別被命為 Der p 1 及 Der f 1 , Eur m 1 及 Blo t 1; 第二群(group 2)過敏原均與人類副睪蛋白(epididymal protein)有分子相似性，被命為 Der p 2 及 Der f 2；第三群(group 3)過敏原均具有 trypsin 之特性，分別被命為 Der p 3 及 Der f 3[(15)]。如果一過敏原有同源異構體(isoallergen)存在(大於 67% 分子序列相似性)，則於阿拉伯數字後再加上小數點及兩碼數字，如 Amb a 1.01，Amb a 1.02；而如果同源過敏原間只有幾個胺基酸之差別，則於小數點後再加兩碼(變四碼)，如 Der p 1.0101、Der p 1.0102、Der p 1.0103 來做區分[(15)]。

只有可與 5%以上或至少 5 名以上病患之 IgE 結合的過敏原，才可以被登錄於此 WHO/IUIS 過敏原資料庫[(23)]。而一過敏原若能與 50% 以上之病患的血清 IgE 反應，則稱為主要過敏原(major allergen)；若僅能與少於 50%之病患的血清 IgE 反應，則稱為次要過敏原(minor allergen) [(24)]。

1.3 昆蟲過敏

昆蟲自古埃及時代便已知道是一重要的過敏原。 昆蟲的屍體殘骸可以變成灰塵的一部分, 如蟑螂，成為吸入過敏原而引起過敏性氣喘或過敏性鼻炎[25-30]；或是藉由叮螫(sting) 或叮咬(bite)將其毒液(venom)或唾液(saliva)注入人體而引起過敏反應。

叮人的昆蟲依其叮的方式不同，又可分為兩大類：

(一) 叮螫昆蟲(Sting insects)：膜翅目昆蟲(Hymenoptera)，如胡蜂科(Family of Vespidae, wasps, yellowjakets, hornets)、蜜蜂(*Apis mellifera*, honey bee)、 紅火蟻(*Solenopsis invicta*, red imported fire ant)等，是以腹部尾端的毒針叮螫(sting)，將其毒液(venom)注入人體， 而造成毒性或過敏反應[34]。

(二) 叮咬昆蟲(Biting insects)：雙翅目(Diptera)，如蚊科(Family of Culicidae, mosquitoes)、蠓科(Family of Ceratopogonidae, midge)；半翅目(Hemiptera)，如臭蟲科(Family of Cimicidae,bed bugs)，及蚤目(Siphonaptera, fleas)等昆蟲，則是以口器叮咬(bite)，將其唾液(saliva)注入人體而引起反應[31-33]。

對昆蟲叮咬或叮螫引起的反應, 因被叮咬個體的不同,而有很大的差異。有些反應是毒液引起的毒性反應，但絕大多數的反應則是個體對毒液或唾液過敏而引起的免疫反應。被叮咬之個體出現的反應，

可由輕微的局部紅腫，到嚴重、甚至致命的過敏性休克都有可能[34-36]。Yocum 等人於 1994 年報告昆蟲叮咬過敏在美國佔因過敏性休克至急診求醫患者的 14%[37]，而 Mullins 等人於 2003 年報告昆蟲叮咬過敏在澳洲佔因過敏性休克至急診求醫患者的 20%[38]，昆蟲叮咬過敏可說是一不容輕忽的公衛問題。除了可能致命以外，即使對昆蟲叮咬過敏反應只是局部反應，往往也會使患者之戶外活動因此大為受限，對患者之生活品質產生極大的影響[39;40]。

目前已知膜翅目昆蟲叮螫後之大塊局部反應型(large local reaction) 及過敏性休克(anaphylaxis)均為免疫球蛋白 E 媒介，且透過減敏治療可以大幅改善，甚至治癒此種過敏反應[41;42]。

而蚊子叮咬後引起的紅腫膨疹反應，也是一免疫球蛋白 E 媒介之過敏反應，且免疫球蛋白 G 也有參與。 對蚊子叮咬有嚴重反應的個體，其血中的唾液特異型 IgE 與 IgG(尤其是 IgG_1 及 IgG_4)明顯升高，且其淋巴球與蚊唾液蛋白一起培養時，會有異常增生情形，所以是一個免疫反應引起的過敏反應，且已知其過敏原為蚊類唾液中的蛋白質[43-46]。

至於其他的叮咬型昆蟲(biting insect)，錐鼻蟲(Triatoma, kissing bugs)是美洲最常引起過敏性休克的叮咬昆蟲。其唾液中的主要過敏原為一 20kDa 之蛋白質，也已經被鑑定且選殖出來[47]。除錐鼻蟲之外，根據

美國過敏氣喘臨床免疫學會之登錄[33]，132 名叮咬型昆蟲叮咬後發生全身型過敏性反應者，肇因昆蟲還包括蚋(blackfly)、 馬虻(horsefly)、鹿虻 (deerfly)、蚊類(mosquito)、庫蠓(Culicoides)、跳蚤(flea)等。

1.4 叮螫與叮咬昆蟲過敏原

膜翅目昆蟲(Hymenoptera)毒液的過敏原,根據研究多半含三至四種主要蛋白質混合其他較小分子，而且多半具有酵素活性。最常見之膜翅目昆蟲主要過敏原為其毒液(venom)中的磷脂酶(phospholipase)及透明質酸酶(hyaluronidase) [48-50] 。

蚊類之唾液過敏原是叮咬昆蟲(biting insect)中被研究較為透徹的。目前已知蚊類唾液中至少有 30 種以上之蛋白質[45]，包含 lysozyme、醣解酶 glucosidase，及抗凝血物質 factor Xa 及 apyrase 等。Peng 等人於 1997 年使用總合過敏病患血清(pooled patient sera)以免疫轉漬法研究全球 10 種不同品種的蚊子唾液過敏原，發現不同品種之蚊類唾液分別含 3 至 16 種過敏原[51]，且有些過敏原是共同的，但也有些過敏原只存在於某些特定品種的蚊類中[52]。以埃及斑蚊(*Aedes aegypti*)為例，其唾液中有 31 個唾液蛋白質之 cDNA 已被定序出來儲於 Genebank 資料庫中[53]，且其中有 12 種唾液蛋白質已知會對人類引起過敏反應[43]。其中 Aed a 1 (68kDa apyrase)[47]， Aed a 2 (37kDa female-specific D7 protein)[54]，Aed a 3 (30kDa 蛋白質，生物活性目前

不明)[43]及 Aed a 4(67kDa α-Glucosidase)[55]已有純化之重組過敏原產生。庫列蚊(*Culex quinquefasciatus*)唾液則已知有 35.5-，32.5-與 22.5 kDa 三種過敏原，且 22.5kDa 之過敏原多半只對兒童會引起過敏反應[56]。

以蜜蜂或黃蜂等膜翅目昆蟲(Hymenoptera)的毒液過敏原來做減敏治療已被證實療效極佳且可明顯改善病患之生活品質[57]。Srivastava 等人 2007 年也發表以雙盲對照之人體臨床試驗以庫列蚊之粗粹蛋白來給嚴重蚊子叮咬過敏的病患做減敏治療可大幅改善蚊子叮咬過敏的症狀[58]。

1.5 重組過敏原的臨床運用

前面提到，臨床上診斷 IgE 媒介之過敏病，主要是靠過敏原皮膚試驗或檢驗病患血液中的過敏原特異型 IgE。傳統上，臨床醫師均使用天然來源之過敏原粗萃取物作為診斷的工具,，而治療過敏病之減敏治療，也是使用粗萃取物作為減敏疫苗。但天然來源之過敏原粗萃取物，常有批次間的變異(batch-to-batch variation)，過敏原蛋白質含量不穩定，含有許多不相關的蛋白質，不容易標準化， 有時還有被其他過敏原或細菌內毒素污染的顧慮, 有些過敏原使用傳統粗萃取法又不易萃出[59;60],使得臨床使用起來,往往有許多不便與不足之處。

過去十餘年來，利用重組 DNA 之基因工程技術來選殖(clone)及製

造純化重組過敏原，使得臨床醫師及研究人員有了大量高度純化的過敏原蛋白質可供研究及臨床使用[61-65]，而以重組過敏原做為減敏疫苗以治療過敏疾病之臨床試驗也陸續進行中[66;67]。

1.6 台灣鋏蠓

1.6.1 台灣鋏蠓之分類

台灣鋏蠓(俗稱小黑蚊)是一種體型微小(1-1.5 mm)的吸血昆蟲，雖然俗稱為小黑蚊，但台灣鋏蠓並不是蚊子，而是雙翅目(Diptera) 蠓科(Ceratopogonidae) 鋏蠓屬（Forcipomyia）蠛蠓亞屬(Lasioheles)之微小昆蟲 。台灣已有記錄的蠛蠓亞屬昆蟲共二十四種[68]，其中只有二種會吸食人血，即台灣鋏蠓 (*Forcipomyia taiwana*) 及三地鋏蠓 (*Forcipomyia anabaenae*)，其他蠛蠓則分別有其專一性之宿主動物，如好牛鋏蠓與叮牛鋏蠓只叮咬水牛而趨光鋏蠓則叮咬蟾蜍。台灣鋏蠓分佈於海拔1-200公尺之平地，最為常見。而三地鋏蠓則分佈於200至850公尺之較高海拔險崖山地，如台灣屏東縣之三地門[68]。

1.6.2 台灣鋏蠓之形態

台灣鋏蠓根據孫、陳、葉等人之研究[69-71]，其卵成紡錘型，長約0.3 mm，最寬處約 0.083mm；卵殼為黑色，無明顯花紋，散產於孳生場所。孵化時卵殼自末約三分之一處斜裂，幼蟲孵化後之卵殼呈拖鞋狀。

初孵化幼蟲約 0.35 mm，體呈透明，老熟幼蟲體長約 2.5-2.7 mm。除前胸及最後一節有小鉤狀之偽足外其餘各節均無足。

台灣銳蠓之蛹為裸蛹，雌者長約 1.9mm，雄者長約 2.1 mm。初蛹化時為淺褐色而後顏色漸深。

雌雄成蟲大小差不多，體長約 1-1.5 mm，頭黑色，觸角及口器深褐色，觸角十四節，基節較大，二至九節為念珠狀，十至十四節則明顯延長。成蟲有翅一對，後翅退化為平均棍。

1.6.3 台灣銳蠓之分佈與生態

1913 年日本昆蟲學家素木得一(Shiraki) 首先提出有關在台灣中部發現台灣銳蠓((*Forcipomyia taiwana*) 之報告[72]。 台灣銳蠓在台灣本島台北郊區、桃園、新竹、台中、南投、彰化、雲林、嘉義、台南、花蓮，宜蘭等縣均有發現[73]，而台灣中、南部及花蓮等縣市尤其嚴重。中國大陸則在山東、湖北、湖南、貴洲、福建、四川、廣東、廣西等省有報告其蹤影[71]。

與蚊子類似，台灣銳蠓只有雌蟲才會吸血。台灣銳蠓雌蟲必須吸血之後才能產卵，雌蟲產卵於陰暗潮濕生有青苔或藍綠藻之土壤表層，其幼蟲成長於潮濕陰涼之山腳、樹林、竹林、茶園、檳榔園及住家房屋四周枝葉茂盛之樹下或庭院花園靠牆陰濕部份[68;71]。台灣銳蠓於實驗室飼養由卵至成蟲需時約14～26 天，其中幼蟲期較長約6～18

天。在自然界因受氣候因子，地理環境與食物之調適，其生長速度可能會比室內飼養之日數為短[69;70]。

台灣鋏蠓雌蟲主要在白天吸血， 陳錦生等人以人體誘集法調查，臺灣鋏蠓之日間吸血活動自上午八時開始逐漸增多，至下午兩點至三點間達最高峰，此後漸漸減少，至黃昏時則完全消失[71;74]。季節之消長根據陳錦生 1980 年之論文於花蓮地區 1976 至 1979 年連續四年間之調查，自元月份起，該蟲族群逐漸上升，至七月份達最高峰，八月至十二月間族群則急劇下降。不同年份間，以日照時間及雨量對該蟲族群數目影響最大[71]。1994 年莊益源等人研究南投地區臺灣鋏蠓之季節消長，則自三月起逐漸增多，六至八月間達到最高峰，十一月至二月間則極少[75]。近年來在台灣的都市、郊區、山區、風景區及都會公園皆發現有台灣鋏蠓，且有繼續漫延之趨勢[76]。

1.6.4 台灣鋏蠓之為害

台灣鋏蠓雌蟲在白天會成群攻擊身體暴露之部位，引起敏感個體劇烈瘙癢及紅腫。2008 年羅偉誠等人雖有報告於台灣鋏蠓雌蟲體內檢出 B 型肝炎(HBV)之病毒訊號[77]，但目前在台灣並不像其他蚊科昆蟲有直接傳染疾病的報告。但其叮咬後所產生的奇癢難耐仍然使其成為重要的騷擾性昆蟲。某些風景區，如台中的大坑山區，台灣鋏蠓肆虐的程度，甚至使遊客為之卻步，嚴重影響當地之觀光業、農業及建

築業。且此蟲因為體型微小，一般住家之紗窗紗門並無法阻絕其進入室內，且施以化學藥劑也僅能短暫減少其數目，數週即回復原有數目，成效不佳[78-80]。且此蟲生命力強，而其蛹浸水後雖然會造成羽化率降低，其幼蟲在大雨之後短期淹水似乎並不會造成幼蟲大量死亡[81]，是台灣吸血昆蟲中，最難防治的一種。

第二章 研究目的

台灣蛺蠓叮咬會引起嚴重反應雖然已被當地人及遊客廣為抱怨，我們在台中榮民總醫院過敏門診也發現不少台灣銳蠓叮咬過敏之患者。但文獻上尚未有任何關於台灣蛺蠓過敏之臨床表徵及其過敏反應免疫機轉之系統性研究，對引起過敏反應的過敏原也完全不了解，更遑論有標準的檢驗方法及減敏疫苗可供臨床使用。

本研究由台灣銳蠓叮咬過敏反應的臨床表徵與流行病學調查為研究出發點，鑑定與選殖其主要過敏原，再進而鑑定純化重組過敏原之免疫特性，並建立間接體內(*ex vivo*)台灣銳蠓過敏實驗模型，以協助釐清其過敏反應之免疫機轉，。

希望本研究的成果，純化之重組過敏原除了將來可以用來作為診斷台灣銳蠓過敏的檢驗試劑以外，也可能可以用來做為治療台灣銳蠓過敏的減敏疫苗。而免疫機轉的研究，則希望能找到最合適的藥物來治療這種惱人的過敏病。

第三章 台灣鋏蠓過敏之流行病學與臨床表徵

3.1 引言

叮螫(sting)昆蟲，如蜜蜂(honeybee, *Apis mellifera*)、黃蜂(wasp,*Vespula germanica*)、大黃蜂(hornet,*Vespa crabo*)等從古埃及時代便已知是人類之重要過敏原。文獻中最早記載，可以溯及公元前2641 年，埃及法老王 Menes 因為被黃蜂叮螫以後發生過敏性休克而身亡，是文獻中第一個死於過敏病例[82]。在古代巴比倫法典中(約公元前二世紀至公元後三世紀)也有被黃蜂叮而致死的記錄[83]。人類對昆蟲叮螫或叮咬的過敏反應，可由輕微之局部癢腫到致命的過敏性休克均有可能，至今歐美國家每年仍有百萬分之 0.1 至 0.5 人因被膜翅目昆蟲叮螫後發生嚴重過敏性休克而喪命[84]，由古埃及時代至二十一世紀仍持續是一個重要的醫療課題。

叮咬昆蟲(biting insect))雖不像叮螫昆蟲(sting insect)那麼常引起致命的過敏反應，但叮咬後之紅腫劇癢仍會造成過敏者極大之不適與生活品質之下降。台灣鋏蠓俗稱小黑蚊，並不是真正的蚊子，而是一種體型微小(約 1-1.5mm 長)的吸血蠓科昆蟲，常造成被叮咬者局部紅腫及劇癢，在台灣全島近郊及中國大陸南方均可見其蹤影影。自 1913 年素木得一在台灣中部首先發現台灣鋏蠓至今已經將近一世紀，但近

年因環境之變遷使得台灣近郊地區，如台中大坑地區之台灣鋏蠓族群暴增，使其危害反有愈加嚴重之趨勢[85]。雌鋏蠓在白天吸人血，常造成過敏者被叮咬處局部紅腫及劇癢，甚至持續數月不消。雖然本地過敏科及皮膚科醫師注意到小黑蚊叮咬後之嚴重反應已經有一段時間, 但除黃等人於 2000 年有一小規模之研究外[86]，之一直未再有關於台灣鋏蠓過敏之臨床與過敏原系統性較大規模之相關研究。本章將就台灣鋏蠓過敏之流行病學及臨床表徵做研究。

3.2 材料及方法

3.2.1 採集台灣鈸蠓雌蟲

我們使用東海大學生命科學系陳錦生老師實驗室設計之捕蠓器(如圖一) ，以人體誘集法活捉台灣鈸蠓雌蟲(如圖二 A、B) ，採集地點包括台中大坑山區、新竹寶山水庫山區及南投縣竹山鎮秀林里，由野外採集回來之新鮮活蟲體立即置入-70℃冰箱冷凍保存。

3.2.2 受試者

本研究內容均經台中榮民總醫院人體試驗委員會審核通過。總共 372 名曾被台灣鈸蠓叮咬之台中榮民總醫院門診患者及台中大坑中台科技大學學生接受護理師問卷訪查。問卷內容包括被台灣鈸蠓及家蚊叮咬後詳細之局部及全身反應、反應持續時間、其他合併之過敏病史與自體免疫病史。受試者若同意， 且簽署書面人體試驗同意書後， 則予以施行皮膚試驗及抽血。

在被叮咬一小時內發生之反應定義為立即型反應 （immediate reaction），6 小時以上才發生之反應定義為延遲型反應 （delayed reaction）。

3.2.3 備製台灣鈸蠓全蟲粗萃取物

約 1000 隻雌蟲經研缽磨碎，溶於 5 毫升 PBS 緩衝液（pH 7.4），於 4℃下超音波震盪(Branson，USA)均質化 30 分鐘後，經 8000g 離

心 15 分鐘。取出上清液後，經 0.45 μm 過濾器，定量蛋白質濃度後，分裝於-70℃冰箱保存。

蛋白質濃度的測定，是採用 Bradford 1976 年提出之方法[87]，將 Coomassie brilliant G250 染劑 (Bio-Rad) 和待測物混合，作用 5 分鐘後，利用光譜儀 (Hitachi, Japan) 測量波長 595nm 的吸光值，以不同濃度 Bovine Serum Albumin (Bio-Rad, CA, USA)作出定量標準濃度曲線，以內插法算出待測物蛋白質濃度。

3.2.4 過敏原皮膚試驗及病患血清檢體之採集

台灣鋏蠓粗萃取液經 0.22 μm 過濾器(Millipore)除菌處理後，以 50% PBS-glycerol 為溶劑配製不同濃度皮膚試驗試劑(100, 200, 400 μg/ml)；histamine (1mg/ml, ALK-Abelló, Denmark) 和 50%PBS-glycerol 分別為陽性(positive control)和陰性對照(negative control)試劑。以無菌拋棄式之 Sharp Test ® (Greer Laboratories, Lenoir, NC, USA) 皮膚試驗針實行皮膚試驗。皮膚試驗由護理師或醫師依照 Sharp Test ®廠商建議之標準的方法進行，反應 20 分鐘後判讀結果。皮膚反應之膨疹(wheal)及紅暈(erythema)若比陰性對照大 3mm 以上, 但比陽性對照之 1/2 直徑小, 則為 1+； 大於陽性對照之 1/2 直徑但小於陽性對照為 2+；與陽性對照同等大小為 3+；大於陽性對照且有偽足者為 4+。[88]

3.2.5 以酵素聯結免疫吸附法(ELISA)測定台灣鋏蠓專一型免疫球蛋白 E

我們以 checkboard titration 方式決定抗原及結合物(conjugate)之理想濃度。[89]台灣鋏蠓粗萃取液 3μg/well 以鹼性 coating buffer (pH9.6)被覆(coating)於 96 孔盤上，於 4℃ coating 隔夜。以 1% 山羊血清(Sigma, San Louis, USA)blocking 兩小時後，以 1:10 稀釋之病患血清為初級抗體，於 4℃反應隔夜，再以 goat anti-human- IgE (Allergopharma, Reinbek, Germany)鹼性磷酸酶為二級抗體於室溫下反應兩小時後, 加入 substrate , ρ-nitrophenyl phosphate (ρ-NPP) 及 AP buffer (Sigma, San Louis, USA) 進行呈色 45 分鐘。終止反應後以 Microplate Absorbance Reader(TECAN，Austria)於析光度 405nm 下讀取光學密度(optic density, O.D.)。標準梯牧草(timothy grass , *Phleum pretense*)過敏原(Allergopharma, Reinbek, Germany)與參考血清之反應(0.35、3.5、及 17.5 kU/l)作為陽性參考標準曲線。30 名無台灣鋏蠓過敏反應者之血清作為陰性對照組。

3.3 結果

3.3.1 台灣鋏蠓叮咬反應的類型及發生率

在 372 位曾被台灣鋏蠓叮咬之受訪者(153 名男性，219 名女性，平均年齡 38.59±15.25 歲，range 1-80 歲) 中，40.9% (152/372)在被台灣鋏蠓叮咬後並無任何不適反應已有免疫耐受產生。59.1% (220/372) 被台灣鋏蠓叮咬後會產生過敏反應(表一)。這 220 個台灣鋏蠓過敏者中，14% (30/220)僅有立即型皮膚反應，於被叮咬一小時內，產生局部明顯的紅腫膨疹，其大小可由直徑 2 公分至大於 10 公分以上，約 1-2 小時症狀即會消失，我們將之稱為純立即型反應(solely immediate reaction) (圖三 A)；43% (95/220)則是在被叮的當下毫無感覺，6-24 小時後才產生劇癢的延遲型反應，而於被叮咬 6-24 小時(部份可到 48 小時)後產生劇癢之丘疹或水泡，有些人之患處中央還會壞死，持續數週至數月方消，我們將之稱為純延遲型反應(solely delayed reaction) (圖三 B)；43%(95/220)除立即型反應外還合併延遲型反應(圖三 C)。在我們的研究族群裡，並未有叮咬後發生呼吸道過敏反應或過敏性休克的報告。

患者之年齡、性別、是否有過敏體質或自體免疫疾病與是否發生台灣鋏蠓過敏並沒有統計上之差別(P>0.05)[90]。

3.3.2 台灣鋏蠓粗萃取液皮膚試驗之結果

共 21 位台灣鋏蠓過敏者願意接受皮膚試驗及抽血。於簽署受試者同意書後，我們依照 3.2.4 所敘之方法，幫患者施行皮膚試驗。12 名立即型反應之患者皮膚試驗對 200 μg/ml 及 400 μg/ml 之台灣鋏蠓粗萃取液均為陽性反應 (圖四 A), 9 名純延遲型反應患者及 10 名無過敏者(data not shown) 對 100 μg/ml, 200 μg/ml 及 400 μg/ml 之台灣鋏蠓粗萃取液均為陰性反應，結果如表二。顯示台灣鋏蠓叮咬之立即型反應可用過敏原皮膚試驗於人體複製(reproduce)之。

3.3.3 台灣鋏蠓專一型免疫球蛋白 E 之測定

21 名接受皮膚試驗之台灣鋏蠓過敏者同時也抽血測定血中之台灣鋏蠓專一型免疫球蛋白 E。如圖四 B 所示，12 名有立即型反應之患者其血清中之台灣鋏蠓專一型免疫球蛋白 E 為 17.75±6.72 kU/l，較 9 名純延遲型反應患者血清中之台灣鋏蠓專一型免疫球蛋白 E (3.58±2.84 kU/l，P=0.005)顯著偏高；無過敏反應者血清中之台灣鋏蠓專一型免疫球蛋白 E 則為 2.71±1.69 kU/l，與純延遲型反應患者並無統計上之顯著差異。顯示台灣鋏蠓叮咬之立即型反應為一 IgE 媒介之過敏反應，而非唾液之毒性反應 。

3.4 討論

吸血蠓叮咬在動物，如馬[91-93]、羊[94]、牛及驢[95]均有報告引起所謂「季節性過敏性皮膚炎」(seasonal allergic dermatitis，sweet itch)的病例，但在人類則無有關吸血蠓叮咬過敏的相關研究。我們的研究顯示，超過半數被叮咬的人類個體(59.1%)會對台灣鋏蠓叮咬有不同程度的過敏反應，可見台灣鋏蠓過敏的確是一常見且重要之公共衛生問題。

我們的研究顯示，台灣鋏蠓叮咬過敏有兩種臨床型態 1)立即型：患者於叮咬 1 小時內產生局部大塊紅腫劇癢反應 2)延遲型：患者於叮咬 12 至 24 小時後生劇癢之丘疹及水泡,甚至皮膚潰爛。此種延遲型皮膚過敏反應往往持續數週甚至數月方消,造成患者極大困擾。患者可以只有立即型或延遲型反應，也可能兩種型合併發生。有立即型反應者，不管是純立即型反應或是還有合併延遲型反應，以台灣鋏蠓粗粹物做過敏原皮膚試驗會有陽性反應，且血清中之台灣鋏蠓專一型免疫球蛋白 E 也顯著偏高；但純延遲型過敏反應之患者以台灣鋏蠓粗粹物做過敏原皮膚試驗均為陰性反應且血清中之台灣鋏蠓專一型免疫球蛋白 E 也極低，顯示這兩種台灣鋏蠓叮咬其免疫機轉可能不相同。由患者顯著偏高的台灣鋏蠓專一型免疫球蛋白 E，我們推論免疫球蛋白 E 為媒介立即型台灣鋏蠓過敏之重要分子，但似乎並非延遲型過敏

反應之主要分子(key molecules)。由 Peng 等人過去的研究我們知道蚊科叮咬之過敏為免疫球蛋白 E 媒介[45]而其延遲型反應則為淋巴球媒介，且在小鼠模型顯示為一 Th2 過敏反應[96]。 究竟這兩種不同型態之台灣銳蠓叮咬過敏反應其免疫機轉為何，我們決定更進一步做探討。

第四章 台灣鋏蠓過敏免疫機轉之研究

4.1 引言

由前一章之台灣鋏蠓過敏流行病學調查結果，我們得知台灣鋏蠓過敏有兩種臨床型態：1)立即型：患者於叮咬 1 小時內產生局部大塊紅腫劇癢反應 2)延遲型：患者於叮咬 12 至 24 小時後生劇癢之丘疹及水泡,皮膚病灶中心甚至出現潰瘍，如 Arthus 樣反應。此種延遲型皮膚過敏反應往往持續數週甚至數月方消，比起速來速退的立即型過敏反應，可說給患者帶來更大的困擾。而我們前一章之研究也顯示，59.1%被台灣鋏蠓叮咬者會產生過敏反應，而純立即型反應只佔所有台灣鋏蠓過敏者之 14%，純延遲型反應或立即型合併延遲型反應者佔了台灣鋏蠓過敏者之 86%。但此一佔病患臨床症狀大宗且更為困擾之台灣鋏蠓叮咬延遲型過敏反應之免疫機轉卻一直沒有相關研究。除了少數蚊子過敏之研究可供參考外[45;97;98]，文獻中幾乎沒有吸血蠓叮咬過敏免疫機轉之研究。

本章之研究，我們將比較立即型與純延遲型台灣鋏蠓叮咬過敏者過敏原專一型免疫球蛋白 E 及 G 反應之不同，並以 *ex vivo* 模型探討延遲型台灣鋏蠓叮咬過敏者之周邊單核細胞在與台灣鋏蠓粹取蛋白質共同培養後細胞及細胞激素的變化。

4.2 材料及方法

4.2.1 採集台灣鋏蠓雌蟲與備製台灣鋏蠓粹取物

同 3.2.1，我們使用東海大學生命科學系陳錦生老師實驗室設計之捕蠓器，以人體誘集法活捉台灣鋏蠓雌蟲，採集地點包括台中大坑山區、新竹寶山水庫山區及南投縣竹山鎮秀林里，由野外採集回來之新鮮活蟲體立即置入-70℃冰箱冷凍保存。

約 1000 隻雌蟲經研缽磨碎，溶於 5 毫升 PBS 緩衝液（pH 7.4），於 4℃下超音波震盪(Branson，USA)均質 30 分鐘後，經 8000g 離心 15 分鐘。取出上清液後，經 0.45 μm (皮膚試驗用)或 0.22 μm(細胞培養用) 過濾器，以 Bradford 法(Bio-Rad, Hercules, CA，USA)定量蛋白質濃度後，分裝於-70℃冰箱保存。

4.2.2 受試者

本研究內容已經台中榮民總醫院人體試驗委員會審核通過。受試者之來源有二：1)因台灣鋏蠓叮咬過敏至台中榮民總醫院過敏免疫風濕科門診就診之病患 2)經本研究徵求受試者之公告得知自動前來者。曾被台灣鋏蠓叮咬之自願受試者經護理師問卷訪查，記錄被台灣鋏蠓及家蚊叮咬後詳細之局部及全身反應、持續時間、其他合併之過敏病史與自體免疫病史後，由過敏科專科醫師陳怡行檢查

其被台灣鋏蠓叮咬之皮膚病灶並根據前章所述之定義予判定台灣鋏蠓叮咬過敏之分型。受試者同意且簽署書面人體試驗同意書後，則以 200μg/ml (in 50% PBS-glycerol)之台灣鋏蠓粹取物施行皮膚試驗及抽血。Histamine (1mg/ml, ALK-Abelló, Denmark) 和 50%PBS-glycerol 分別為陽性(positive control)和陰性對照(negative control)試劑。以無菌拋棄式之 Sharp Test ® (Greer Laboratories, Lenoir, NC, USA) 皮膚試驗針實行皮膚試驗。皮膚試驗由護理師或醫師依照 Sharp Test ®廠商建議之標準的方法進行，反應 20 分鐘後判讀結果。膨疹與紅暈反應比陰性對照(negative control)大於 3mm 以上為陽性反應。皮膚試驗後受試者接受抽血 30 至 50 毫升。

受試者依其被台灣鋏蠓叮咬後之臨床表徵及皮膚試驗反應結果分為三組 1)立即型：被台灣鋏蠓叮咬後有立即型反應者，不論是否合併有延遲型反應 2)純延遲型反應者 3)對照組：被台灣鋏蠓叮咬完全無反應，已產生耐受者。

4.2.3 台灣鋏蠓專一型免疫球蛋白 E, G 及免疫球蛋白 G 亞型的測定

我們將台灣鋏蠓粗萃取液 3μg/well 以鹼性 coating buffer (pH9.6) 被覆(coating)於 96 孔盤上，於 37℃ 孵育兩小時。以下每步驟之間均以 washing buffer 經過 3 次 washings。Washings 後以 1% 山羊血清

(Sigma, San Louis, USA)於室溫 blocking 兩小時後，以 1:10 稀釋之病患血清為初級抗體，於室溫反應 2 小時。Washings 後再以 mouse anti-human- IgE (BD Pharmigen, San Diego,CA,USA) 或-IgG, -IgG_1, -IgG_2, -IgG_3, -IgG_4 抗體 (Zymed Laboratories, South San Francisco,CA, USA) 於室溫下反應兩小時後, 加入 substrate ρ-nitrophenyl phosphate (Sigma, St. Lous, MO, USA)。終止反應後以 Microplate Absorbance Reader (TECAN，Austria)於析光度 405nm 下讀取光學密度(optic density, O.D.)。

4.2.4 周邊單核球之製備

受試者之周邊單核球 (peripheral mononuclear cells，PBMC)由加有肝素之靜脈血以 Ficoll-Paque (Pharmacia, Uppsala, Sweden) 離心分離之。以 PBS 經過三次洗滌後，將細胞重新懸浮於含 RPMI (Sigma)、2g/ml 重碳酸鈉、mM L-glutamine、5mM HEPES 緩衝液、200U/ml penicillin、200μg/ml streptomycin 與 10%加熱去活受試者自體血清之培養基中。

4.2.5 以 MTT assay 測量淋巴球增生反應

$2x10^5$ 受試者之周邊單核球，分別與 1) 僅有培養基 2) 0.2μg/ml Concanavalin A (Con A) 3) 0.3 μg/ml 台灣鋏蠓萃取物 4) 3 μg/ml 台

灣鈸蠓萃取物 5) 30 μg/ml 台灣鈸蠓萃取物於培養基液中共培養 24 及 48 小時後, 加入 20μl (5mg/ml) tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) 反應一小時後, 以 dimethyl sulfoxide(DMSO) 將結晶溶解並以 microtiter plate reader (TECAN)於 570nm 下測其吸光值(optic density, O.D.)。MTT Assay 或稱 Tetrazolium assay 是一般常用的細胞存活試驗方法。原理為 MTT 黃色水溶性染料可被細胞內粒腺體中的去氫酶 (dehydrogenase)代謝，將 tetrazolium ring 切斷還原成紫色不溶性沈澱物 formazan 堆積在細胞中，被 DMSO 溶解。因活細胞才具有活性的粒腺體酵素，故所測得的吸光值會與活細胞數量成正比關係，因此可利用 formazan 產量的多寡來評估淋巴球的增生率。

實驗之數值以 Stimulation index 來表示。其計算方法為：與小黑蚊萃取蛋白共培養細胞之 O.D. 570nm 值 ÷ 同一時間點僅有培養基培養細胞之 O.D. 570nm 值[99]。

4.2.6 細胞培養

在 12x75mm BD Falcon™ 之細胞培養試管(BD Biosciences, FranklinLakes, NJ, USA)中每管植入 $1x10^6$ 之受試者周邊單核球於 1ml 之 1)僅有培養基 2)加入 0.2μg/ml Con A 或 3) 3μg/ml 台灣鈸蠓粹取物共同培養 24、72 及 144 小時後加入 50 ng/ml Phorbol

12-Myristate-13-Acetate (PMA)，再培養 24 小時後於 1500rpm 下離心 10 分鐘後收取細胞與上清液做進一步分析。細胞於上清液取出後置入 RNAlater (Ambion, Foster City, CA, USA)保存以便抽取核糖核酸(RNA)。除去細胞之培養上清液凍存於-70 度冰箱中待進一步分析。

4.2.7 抽取核糖核酸(RNA)及合成互補去氧核糖核酸(cDNA)

4.2.6 培養所得之周邊單核球以 RNAzol (Invitrogen, Carlsbad, CA, USA)試劑依原廠說明書操作方式抽取核糖核酸。核糖核酸之純度以分光光度計(spectrophotometer, BECKAMAN DU7400)測定 260nm 與 280nm 吸光度之比值來測定。互補去氧核糖核酸(cDNA)以下列方式合成：1μg 全核糖核酸加入 20μl 含 2.5μM oligo-dT 引子、1mM 三磷去氧核糖核甘(deoxyribonucleoside triphosphates, dNTP)、5mM 二硫蘇糖醇(dithiothreitol)、40U RNaseOUT 核糖核酸脢抑制劑與 15U 之 ThermoScript 反轉錄脢之混合反應液共同反應。 反轉錄反應於 thermocycler 反應槽(Perkin-Elmer, Irvine, CA, USA)中以攝氏 50 度反應 60 分鐘接著於攝氏 85 度下反應 5 分鐘。

4.2.8 以即時聚合酵素連鎖反應(real-time PCR)檢測細胞激素之訊息核糖核酸

細胞激素訊息核糖核酸之值以 GeneAmp 7000 Sequence Detection System (Applied Biosystem, Uberlingen, Germany)將 4.2.7 合成之互補去氧核糖核酸以 TaqMan PCR 分析之。預製之人類丙型干擾素(interferon-gamma, IFN-gamma)、第十介白質、第六介白質及甲型腫瘤壞死因子(tumor necrosis factor-alpha, TNF-alpha)專一性序列偵測試劑係向 Applied Biosystem 公司訂購。為標準化每一檢體內之核糖核酸量，我們以乙型肌動蛋白(beta-actin)做為控制基因。此系統之每一探針皆有一螢光報告染劑(FAM)結合至其 5'端且有一下游之抑制染劑(quencher dye TAMRA)結合至其 3'端。每 20μl 之 PCR 混合反應試劑中含有 TaqMan 通用 Master Mix 與 AmpliTaq Gold 去氧核糖核酸聚合脢、探針/引子混合液與 50ng 互補去氧核糖核酸。放大反應是在攝氏 95 度反應 10 分鐘後，再經 45 循環之「攝氏 95 度 15 秒—攝氏 60 度 1 分鐘」連鎖反應。閥值循環值(threshold cycle, C(t))，即螢光值升高至可偵測範圍時之反應循環數，是以 GeneAmp 軟體來計算。實驗數值係以台灣鋏蠓粹取蛋白刺激組與僅培養基組與控制基因標準化後之倍數變化(fold change)來表示。

4.2.9 以流式細胞微珠免疫法(Flow cytometric bead immunoassay)測定細胞培養上清液中細胞激素蛋白質

細胞培養上清液以流式細胞微珠免疫法 FlowCytomix (Bender MedSystem, Vienna, Austria)[100;101]同時測定 11 種不同之細胞激素，包括第一介白質乙(IL-1 beta)、第二介白質、第四介白質、第五介白質、第六介白質、第八介白質、第十介白質、第十二介白質 p70、丙型干擾素、甲型腫瘤壞死因子與乙型腫瘤壞死因子。經過最後一次洗滌後，每一反應孔內之檢體以 200µl 之化驗緩衝液使之懸浮、移入 12x17 之聚苯乙烯(polystyrene)管內並以 FACScanTM 流式細胞儀(BD Biosciences, Franklin Lakes, NJ, USA)分析之。 細胞激素的濃度以各細胞激素標準曲線之值換算之。

4.2.10 以 Azocoll 法測定台灣鋏蠓粹取物之蛋白腖活性

我們以 Chavira 等人描述之方法[102]以膠原蛋白 Azocoll (Sigma) 法測定台灣鋏蠓粹取物之蛋白腖活性。在試管 1 至 9 中分別加入 25mg 之蛋白腖受質 Azocoll，接著於 37℃下於試管 1 至 7 中分別加入稀釋於 100mM 鉀磷酸緩衝液(potassium phosphate buffer)之 *Bacillus subtilis* 蛋白腖 subtilisin (Sigma) 1) 0 unit 2)2.4x10^{-3}unit 3)4.8 x10^{-3} unit 4)7.2 x10^{-3} unit 5)9.6 x10^{-3}unit 6)12 x10^{-3} unit 7)1.2 unit。試管 8 加入台

灣鋏蠓粹取物(20μg/ml)，試管 9 加入半純化蟑螂過敏原 Cr PI (12μg/ml，已知具有強蛋白腖活性)。所有試管於 37℃之搖晃水浴槽 (60cycles/min) 內反應 15 分鐘 。將試管由水浴槽取出後立刻以 0.45μm 過濾器過濾，再移至透明 cuvettes 中以 Microplate Absorbance Reader (TECAN，Austria) 於析光度 520nm 下讀取光學密度(optic density, O.D.)。以試管 1 至 7 之吸光值換算成蛋白腖活性畫出標準曲線後，再以內插法計算出台灣鋏蠓粹取物與蟑螂過敏原 Cr PI 之蛋白腖活性。

4.2.11 蛋白腖抑制劑對台灣鋏蠓粹取物誘發 A549 細胞第八介白質分泌之作用

我們以購自台灣新竹市台灣生物資源保存及研究中心 (Bioresource Collection and Research Center ,Taiwan)之呼吸道上皮細胞株 A549 細胞在含 10% 胎牛血清、2mM L-glutamine、100 units/ml penicillin 與 100 μg/ml streptomycin 之培養基 RPMI 1640 中培養。實驗用之 A549 細胞均培養於 6 孔培養盤中至達 80%融合度時始進行實驗。台灣鋏蠓粹取物先分別於 37℃與不同種類及不同濃度之蛋白腖抑制劑反應 15 分鐘後，加入 A549 細胞中，再續予培養 24 小時。離心取出培養之上清液後凍存於-70℃中待進一步分析。實驗中共使用以下三種蛋白腖抑制劑：phenylmethane sulfonyl fluoride (PMSF；濃

度 0.01 至 0.4nM)、E-64 (濃度 5 至 200ng/ml)、及 pepstatin (濃度 5 至 200 ng/ml)。三種蛋白胺抑制劑均購自 Sigma 公司，且使用前均溶於去離子磷酸緩衝溶液中(Dulbecco’s phosphate buffer saline，Bibco Invitrogen, USA) 。

培養上清液中第八介白質之濃度以酵素聯結免疫法試劑套組(Bender)根據原廠使用說明檢測之。

4.2.12 統計分析

所有實驗數據除非另有說明，均以平均值±標準誤(mean±standard error of mean)表示。平均值之差異以 SPSS 10.0 軟體(SPSS Inc. Chicago, IL, USA) 以無母數 Mann-Whitney U test 統計法分析之。P 值小於 0.05 視為有統計上之顯著差異。

4.3 結果

4.3.1 台灣鋏蠓專一型免疫球蛋白 E 與專一型免疫球蛋白與不同叮咬反應之相關性

共有 62 名受試者參與此項研究，我們根據其叮咬後之不同反應予以分為三組，如表三所示。其中 20 名受試者為台灣鋏蠓叮咬後有立即型過敏反應者(可能無延遲型過敏反應或合併延遲型過敏反應），12 名受試者為純延遲型過敏反應者(solely delayed reactions)而 30 名受試者為對台灣鋏蠓叮咬已產生耐受性，叮咬後完全無任何不適者之對照組(non-allergic control)。受試者之血清由靜脈採血後離心分離之並測定其中之台灣鋏蠓專一型免疫球蛋白 E、專一型免疫球蛋白 G、專一型免疫球蛋白 G_1、專一型免疫球蛋白 G_2、專一型免疫球蛋白 G_3 及專一型免疫球蛋白 G_4。立即型過敏反應組受試者之台灣鋏蠓專一型免疫球蛋白 E 之值(O.D.=0.37±0.06，median=0.31，range 0.1~1.08)比純延遲型過敏反應組受試者(O.D.= 0.10±0.01, median=0.10, range 0.05~0.16)及對照組(O.D.=0.07±0.01, median=0.06, range 0.05~0.09)顯著偏高($P<0.05$），如圖五 a 所示。而三組受試者之台灣鋏蠓專一型免疫球蛋白 G 及專一型免疫球蛋白 G 之亞型均無明

顯差異，如圖五 b 所示。

4.3.2 純延遲型過敏反應者之周邊單核球以台灣鋏蠓粹取物刺激後明顯增生

有兩名純延遲型過敏反應過敏反應之受試者於台灣鋏蠓叮咬 24 至 48 小時後接受皮膚切片檢查，於叮咬反應之病灶內可見大量單核之淋巴組織球(lymphohistiocytes)與些許嗜伊紅性白血球之浸潤，如圖六所示。

因延遲型過敏反應患者皮膚病灶之浸潤細胞見有大量單核之淋巴球浸潤，我們決定進一步於試管中測試受試者之周邊單核球以台灣鋏蠓粹取物次激後之反應，以模擬台灣鋏蠓叮咬之延遲型過敏反應(*ex vivo* model)。六名純延遲型過敏反應之受試者及六名無過敏之對照組受試者由靜脈採血後分離其周邊單核球並以不同濃度之台灣鋏蠓粹取物刺激之再以 MTT 法測定其淋巴球之增生反應。我們發現純延遲型過敏反應之受試者其周邊單核球在受到 3μg/ml 台灣鋏蠓粹取物刺激 24 小時(stimulation index=4.83±2.44 vs. 1.02±0.16)及 48 小時(stimulation index=7.22±2.78 vs. 1.24±0.38)後比對照組受試者明顯增生。更高濃度 30μg/ml 之台灣鋏蠓粹取物所得的細胞增生反應與 3μg/ml 並無差異，如圖七所示。

4.3.3 純延遲型過敏反應者周邊單核球以台灣鋏蠓粹取物刺激後特定細胞激素明顯增加

如圖八a、c、e、g所示，純延遲型過敏反應受試者(SDR)周邊單核球比對照組 (NAC)於48、96及168小時刺激後表現較高之丙型干擾素(IFN-γ)、第十介白質(IL-10)、第六介白質(IL-6)與甲型腫瘤壞死因子(TNF-α)訊息核糖核酸。其細胞激素訊息核糖核酸之中位數(median)倍數變化與範圍(range) 如下 (SDR vs. NAC)：

IFN-γ於48h： 4.66 (1.22 to 9.99) versus 2.06 (–1.03 to 4.76), 於96h: 4.89 (–1.44 to 16.68) versus 1.38 (–7.06 to 5.03), 於168h: –1.36 (–4.06 to 1.21) versus –1.03 (–3.94 to 7.57).

IL-10於48h: 3.22 (1.33 to 7.62) versus 0.08 (–2.19 to 1.36), 於96h ：–0.57 (–2.20 to 4.32) versus –2.26 (–35.26 to 10.20), 於168h: 0.02 (–5.37 to 6.19) versus –3.65 (–15.78 to 6.41).

IL-6於48h: 7.63 (–5.98 to 19.43) versus 2.68 (–1.45 to 12.55), 於96h: 2.20 (–2.60 to 29.65) versus –1.69 (–19.4 to 10.03), 於168h: 1.85 (–1.59 to 13.74) versus 1.05 (–1.43 to 6.36).

TNF- α於48h: 1.61 (1.01 to 2.71) versus 1.34 (1.04 to 1.88), 於96h: 0.87 (–1.93 to 2.95) versus –0.30 (–4.26 to 1.82), 於168h: 1.2 (–0.35 to 2.38) versus –0.90 (–4.20 to 2.08).

如圖八b、d、f及h所示，純延遲型過敏反應受試者(SDR)周邊單核球於48、96及168小時刺激後其丙型干擾素(IFN-γ)、第十介白質(IL-10)、第六介白質(IL-6)與甲型腫瘤壞死因子(TNF-α) 蛋白質亦比對照組(NAC)於所有的時間點分泌均增加。且第十介白質於96小時、第六介白質於48小時而甲型腫瘤壞死因子於168小時之刺激後其反應與對照組相比達統計上之顯著意義。其細胞激素蛋白質之中位數（median)與範圍(range) 如下(SDR vs. NAC）：

IFN-γ於 48h： 51 pg/ml (24 – 109) versus 22 pg/ml (5 – 43), 於 96h： 43 pg/ml (29 – 94) versus 21 pg/ml (5 – 31), 於 168h：26 pg/ml (25 – 64) versus 16 pg/ml (5 – 24).

IL-10 於 48h：8 pg/ml (5 – 10) versus 7 pg/ml (2 – 10), 於 96h： 19 pg/ml (16 – 39) versus 7 pg/ml (6 – 8), 於 168h：16 pg/ml (8 – 23) versus 14 pg/ml (4 – 25).

IL-6 於 48h：298 pg/ml (104 – 835) versus 210 pg/ml (35 – 346), 於 96h：340 pg/ml (190 – 866) versus 215 pg/ml (80 – 222), 於 168h： 244 pg/ml (51 – 698) versus 208 pg/ml (16 – 298).

TNF-α 於48h：91 pg/ml (63－263) versus 16 pg/ml (8－42), 於96h：89 pg/ml (67－257) versus 68 pg/ml (10－196), 於168h：213 pg/ml (102－635) versus 87 pg/ml (9－152).

如表四所示，第八介白質與第二介白質兩組受試者無差異。第四介白質、第五介白質、第十二介白質 p70 及乙型腫瘤壞死因子則未能於培養上清液中測得。

4.3.4 台灣鋏蠓粹取物具蛋白腖活性

承以上實驗結果，特別引起我們注意的是，受試者的周邊單核球於受到台灣鋏蠓粹取物刺激時，分泌大量之第八介白質(大於 10000 pg/ml) ，與細胞受到具蛋白腖活性之過敏原刺激之反應非常雷同[103;104]，使我們想進一步探討台灣鋏蠓之叮咬過敏反應，是否與蛋白腖活化受體 (protease activated receptor, PAR) 路徑是否有關。我們於是先測定台灣鋏蠓粹取物中是否有蛋白腖活性且以已知具強蛋白腖活性之蟑螂過敏原 Cr PI 為比對。我們發現可以 Azocoll 法測得台灣鋏蠓粹取物中之蛋白腖活性且台灣鋏蠓粹取物中之蛋白腖活性(8.5 $x10^{-3}$ units)比蟑螂過敏原 Cr PI (6.3 $x10^{-3}$ units)更高，如圖九所示。

4.3.5 特定蛋白腖抑制劑可抑制 A549 細胞台灣鋏蠓粹取物刺激分泌

之第八介白質

A549 細胞被台灣鋏蠓粹取物刺激所分泌之第八介白質可被 E64(木瓜蛋白胺 papain 與半胱氨酸 cysteine 蛋白胺抑制劑)及 pepstatin(天門冬胺酸 aspartic 蛋白胺抑制劑)抑制且其抑制程度與蛋白胺抑制劑濃度呈正相關，而 PMSF (絲胺酸 serine 與半胱胺酸 cysteine 蛋白胺抑制劑)則無法抑制第八介白質之分泌。如圖十所示。

4.4 討論

台灣鋏蠓叮咬過敏在台灣中部是一常見的搔擾性問題。在本研究中我們發現有台灣鋏蠓叮咬立即型過敏反應者其台灣鋏蠓專一型免疫球蛋白 E 顯著偏高，此與過去蚊科叮咬過敏之研究結果相符[45;98]。但 Peng 等人之研究顯示蚊科叮咬立即型過敏反應者其蚊專一型免疫球蛋白 G_1 與專一型免疫球蛋白 G_4 比對照組顯著升高[105]，我們的研究結果卻發現台灣鋏蠓叮咬立即型過敏反應者其台灣鋏蠓專一型免疫球蛋白 G 與其四種亞型與對照組並無差別。台灣鋏蠓專一型免疫球蛋白 G 與專一型免疫球蛋白 G_4 在三組受試者均有明顯上升，與受試者之叮咬反應並無相關性。推論台灣鋏蠓台灣鋏蠓專一型免疫球蛋白 G 與專一型免疫球蛋白 G_4 可能僅與台灣鋏蠓之暴露(exposure)有關

而與是否發生過敏反應並無相關。

Chen 等人曾於一蚊叮咬過敏之小鼠模型發現這些蚊叮咬有延遲型過敏反應之小鼠在被蚊叮咬後第四介白質分泌明顯增加而丙型干擾素之分泌則下降，顯示小鼠蚊叮咬之延遲型過敏反應為一 Th2 免疫反應。但在我們之間接體內(*ex vivo*)台灣鋏蠓過敏之實驗結果卻顯示人類台灣鋏蠓延遲型過敏反應者較對照組分泌較高之丙型干擾素、第十介白質、第六介白質與甲型腫瘤壞死因子，無論是訊息核糖核酸或是蛋白質層次均如此。丙型干擾素被認為是一 Th1 免疫反應之代表性細胞激素而甲型腫瘤壞死因子與第六介白質則主要是先天免疫反應(innate immune response)細胞所釋放出來之細胞素[106;107]。但是因為我們的實驗對象僅有台灣鋏蠓純延遲型過敏反應者，以無台灣鋏蠓過敏者為對照組，缺少台灣鋏蠓純立即型過敏反應者做為另一對照組，故我們的目前的研究結果並無法排除 Th2 免疫反應參與台灣鋏蠓立即型過敏反應之可能性。

甲型腫瘤壞死因子是許多發炎性疾病之關鍵性分子。甲型腫瘤壞死因子參與過敏性疾病，尤其是在頑固型氣喘的角色已有越來越多之證據[108]。但有關甲型腫瘤壞死因子在叮咬或叮螫昆蟲過敏之文獻非常之少，僅有 Carballido 等人於 1992 年報告人類蜂毒 phospholipase A2 特異型 T 細胞株在以過敏原刺激後會分泌甲型腫

瘤壞死因子[109]。Levi-Schaffer 等人於 1998 年亦曾以實驗證明肥大細胞所分泌之甲型腫瘤壞死因子可藉由自體分泌顆粒球巨嗜細胞群落刺激因子延長嗜伊紅性白血球之存活[110]。此一發現似可用來解釋我們的實驗以過敏原台灣銳蠓粹取物刺激台灣銳蠓延遲型過敏反應病患之細胞後，雖未見具嗜伊紅性白血球趨化作用之第五介白質上升，但病患皮膚切片組織學所見之嗜伊紅性白血球浸潤仍有增多之現象，可能與甲型腫瘤壞死因子之明顯上升有關。

第十介白質一直以來被認為是一抗發炎之細胞激素，主要是由調節型 T 細胞所製造分泌。第十介白質可抑制 Th1 與 Th2 細胞之活化與細胞激素的分泌[111;112]。第十介白質也被認為與自然產生之過敏原免疫耐受[113]及減敏治療之療效[114]有關。但第十介白質升高在我們研究的台灣銳蠓延遲型過敏反應者中似乎並不是一個好的個體是否已產生免疫耐受的指標。因為我們的實驗中有延遲型過敏反應者在台灣銳蠓粹取物刺激後比已有免疫耐受無過敏反應的對照組受試者分泌更多的第十介白質。第十介白質升高在台灣銳蠓過敏似乎為反應個體細胞”接觸過敏原”且試圖降低發炎反應的現象，而非已產生免疫耐受的指標。

在我們的研究中，我們也在台灣銳蠓粹取物中測得蛋白脢活性且台灣銳蠓粹取物刺激之第八介白質分泌可被木瓜蛋白脢及天門

冬胺酸 aspartic 蛋白腖抑制劑所抑制，而無法被絲胺酸 serine 與半胱胺酸 cysteine 蛋白腖抑制劑所抑制，可推知台灣鋏蠓粹取物中之蛋白腖不屬絲胺酸與半胱胺酸蛋白腖。至於是否為木瓜蛋白腖、天門冬胺酸蛋白腖或是其他種類之蛋白腖則則需進一步實驗方可確認。由文獻得之，內生胞外之蛋白腖如凝血酶(thrombin)與胰蛋白酶(trypsin)[115]，以及外源之蛋白腖，如蟑螂[116]、塵蟎[117]與黴菌[118]，均可經與呼吸道上皮細胞細胞膜上之受體作用而誘使白血球浸潤進而增強過敏原所引起之發炎反應。也有越來越多證據顯示蛋白腖活化發炎經由蛋白腖活化受體(protease-activated recptor, PAR)在非免疫球蛋白 E 媒介之過敏性呼吸道疾病扮演一極重要之角色[13]。而皮膚 PAR-2 受體之活化也有報告顯示與皮膚之搔癢、發炎細胞之招募及色素沉澱均有相關[119-121]。以我們在台灣鋏蠓粹取物所測得之強蛋白腖活性，我們相信蛋白腖活化發炎在此一劇癢且易留下黑色素沉澱之台灣鋏蠓過敏必定有其重要之角色。但另一我們的研究尚未能解答之疑問，為蛋白腖活化為一不具專一性之受體反應，對台灣鋏蠓叮咬不會過敏者必定有一未知之負向調控機制來造成台灣鋏蠓叮咬之免疫耐受。

總結來說，我們本章的研究顯示台灣鋏蠓立即型過敏反應為免疫球蛋白 E 媒介但並非延遲型過敏反應之主要分子。丙型干擾素、

第六介白質與甲型腫瘤壞死因子均參與延遲型過敏反應，顯示 Th1 細胞與延遲型過敏反應可能有關。蛋白胺活化路徑 (protease-activated pathway)可能也參與台灣鋏蠓叮咬過敏之免疫反應。

第五章 台灣鋏蠓主要過敏原之鑑定

5.1 引言

過敏原是指會誘使免疫細胞製造免疫球蛋白E或與免疫球蛋白E結合(binding)而引起過敏反應的抗原[122]。大部分會與免疫球蛋白E反應的過敏原是蛋白質，通常為具有一碳水化合物支鏈之醣蛋白，但很少數的狀況下，純碳水化合物也可以成為一過敏原[16;123]。根據WHO/IUIS過敏原資料庫之定義，只有可與5%以上或至少5名以上病患之免疫球蛋白E結合的過敏原，才可認定為一新過敏原，可以被登錄於此WHO/IUIS過敏原資料庫[23]。而一過敏原若能與50%以上之病患的血清免疫球蛋白E反應，則稱為主要過敏原(major allergen)；若僅能與少於50%之病患的血清免疫球蛋白E反應，則稱為次要過敏原(minor allergen) [24]。

承第三章與第四章之研究結果，我們知道59.1%被台灣鋏蠓叮咬者會產生過敏反應，而立即型之台灣鋏蠓叮咬過敏反應為一免疫球蛋白E媒介之第一型過敏反應。究竟台灣鋏蠓體內是哪些過敏原與免疫球蛋白E結合後產生人類之過敏反應，而其主要過敏原為何，是本章想要探討的重點。

5.2 材料及方法

5.2.1 採集台灣鋏蠓雌蟲與備製台灣鋏蠓粹取物

同 3.2.1，我們使用東海大學生命科學系陳錦生老師實驗室設計之捕蠓器，以人體誘集法活捉台灣鋏蠓雌蟲，採集地點包括台中大坑山區、新竹寶山水庫山區及南投縣竹山鎮秀林里，由野外採集回來之新鮮活蟲體立即置入-70℃冰箱冷凍保存。

約 1000 隻雌蟲經研缽磨碎，溶於 5 毫升 PBS 緩衝液（pH 7.4），於 4℃下超音波震盪(Branson，USA)均質 30 分鐘後，經 8000g 離心 15 分鐘。取出上清液後，經 0.45 μm (皮膚試驗用)或 0.22 μm(細胞培養用) 過濾器，以 Bradford 法(Bio-Rad, Hercules, CA)定量蛋白質濃度後，分裝於-70℃冰箱保存。

5.2.2 受試者

本研究內容已經台中榮民總醫院人體試驗委員會審核通過。受試者之來源有二：1)因台灣鋏蠓叮咬過敏至台中榮民總醫院過敏免疫風濕科門診就診之病患 2)經本研究徵求受試者之公告得知自動前來者。曾被台灣鋏蠓叮咬之自願受試者經護理師問卷訪查，記錄被台灣

鋏蠓及家蚊叮咬後詳細之局部及全身反應、持續時間、其他合併之過敏病史與自體免疫病史後，由過敏科專科醫師陳怡行檢查其被台灣鋏蠓叮咬之皮膚病灶並根據 3.2.2 所述之定義予判定台灣鋏蠓叮咬過敏之分型。受試者同意且簽署書面人體試驗同意書後， 則以 200μg/ml (in 50% PBS-glycerol)之台灣鋏蠓粹取物施行皮膚試驗及抽血。Histamine (1mg/ml, ALK-Abelló, Denmark) 和 50%PBS-glycerol 分別為陽性(positive control)和陰性對照(negative control)試劑。以無菌拋棄式之 Sharp Test ® (Greer Laboratories, Lenoir, NC, USA) 皮膚試驗針實行皮膚試驗。皮膚試驗由護理師或醫師依照 Sharp Test ®廠商建議之標準的方法進行，反應 20 分鐘後判讀結果。膨疹與紅暈反應比陰性對照(negative control)大於 3mm 以上為陽性反應。

受試者被台灣鋏蠓叮咬後有立即型過敏反應(大部份受試者還合併有延遲型過敏反應)且皮膚試驗為陽性反應者才納入此部份之研究。受試者於皮膚試驗後經靜脈抽血 30 至 50 毫升。

5.2.3 蛋白質電泳分析和免疫轉漬法

台灣鋏蠓粹取物以 Laemmli 的方法[124]經 SDS-PAGE(上層 4%，下層 12%)電泳後，分析其蛋白質成份。再利用半乾式轉漬槽將膠片上的蛋白質轉移至硝化纖維膜(PVDF)上[125]，用 5%脫脂奶粉去除非

專一性結合，經與受試者血清(10 倍稀釋)在 4℃作用隔夜後，以鹼性磷酸酶受質冷光偵測試劑 CSPD (Tropix，Bedford, MA, USA)及冷光增強劑 (Applied Biosystem, Bedford, MA)反應，在 X 光底片顯影並判讀。

5.2.4 蛋白質二維電泳分析

蛋白質二維電泳第一維管柱膠長 14 公分內徑 1.5 毫米(Bio-Rad protean Ⅱ xi 2-D cell system)，內含 1.5% CHAPS (3-[3-(cholamidopropyl)-dimethy-lammonlo]-1-propanesulphonate; $C_{32}H_{58}N_2O_7S$)，0.5% NP-40, 9.2 M urea, 4.5% acrylamide/bis (29.2:0.8), 5% pH 3~10 carrier ampholytes (Bio-Rad, CA, USA)。負極和正極的緩衝液分別是 20mM NaOH 和 10nM H_3PO_4。 第一維管柱膠電泳先以 200 伏特反應 2 小時。其次 500 伏特反應 2 小時，然後 800 伏特 16 小時達總量 14200 伏特-小時。第一維電泳後，將第一維管柱膠以針筒擠出，浸泡在含 2.8% SDS, 72mM Tris-HCl(pH6.8)及微量的 bromophenol blue(BPB) 染劑的緩衝液中 15 分鐘後，置入第二維片膠狀上層。第二維蛋白質電泳的進行同 Laemmli 的方法[124]。二維電泳後之膠片一方面進行銀染(silver stain)， 一方面以立即型過敏反應受試者血清進行免疫轉漬法，以確認免疫球蛋白 E 結合蛋白的正確位

置。

5.2.5 質譜儀分析與胜肽質量指紋鑑定

台灣鋏蠓蛋白質經二維電泳膠及銀染後，精確切下標的蛋白質，經胰蛋白酶進行 in-gel-digestion(Montage In-Gel Digest Kit, Millipore)後，委由台南國立成功大學貴重儀器中心以電噴灑游離子原串聯式質譜儀 (electrospray ionization tandem mass spectrometry, ESI-MS/MS, Finnigan MAT, San Jose, CA, USA) 鑑定過敏原蛋白質身分。利用高效能液相層分儀分離 tryptic 胜肽群，分析每個胜肽片段的離子質量與部分序列訊息。然後與美國國家生物科技資訊中心 NCBI (National Center for Biotechnology Information, nonredundant)蛋白質序列資料庫(http://www.matrixscience.com, Matrix Science)內之資料比對，搜尋符合的可能蛋白質序列。根據該資料庫之定義[126]，若兩蛋白質序列比對之 Mowse scores 大於 67 ($P<0.05$)，則認為有顯著之序列相似性。

5.3 結果

5.3.1 以免疫轉漬法測定過敏原(IgE-結合蛋白)

台灣鋏蠓粗萃取物經蛋白質電泳 SDS-PAGE 分析，至少有 33 種不同之蛋白質組成，其分子量由 10 到 70 kDa，如圖十一。

以免疫轉漬法發現其中有 11 個蛋白質可與台灣鋏蠓立即型過敏反應病患血清中的免疫球蛋白 E　結合，如圖十二(a)，而 22-、24-、35-、36-及 64-kDa 之蛋白質過敏原可分別與 50%、50%、75%、66.7%及 75%之病患血清反應，符合 WHO/IUIS 主要過敏原之定義，如圖十二(b)。純延遲型反應之病患及無過敏之正常者均未有任何之免疫球蛋白 E 結合蛋白反應(實驗結果未附於文中)。

5.3.2 蛋白質二維電泳分析台灣鋏蠓過敏原

台灣鋏蠓粹取物經蛋白質二維電泳後再以台灣鋏蠓立即型過敏反應病患血清做免疫轉漬，顯示 22-、24-、35-、36-及 64 kDa 台灣鋏蠓過敏原之等電位點(Isoelectric point, pI) 分別為 8.7、5.8、8.6、8.7及 5.2，如圖十三所示。

5.3.3 質譜儀分析與胜肽質量指紋鑑定

24-、35-、36-及 64kDa 台灣鋏蠓過敏原經二維電泳膠及銀染後，切下標的過敏原，以電噴灑游離子原串聯式質譜儀鑑定過敏原蛋白質身分。

質譜儀分析與胜肽質量指紋鑑定結果, 24kDa 過敏原與瘧蚊(*Anopheles merus*), 黃粉蟲(*Tenebrio molitor*), 東鄉伊蚊(*Ochlerotatus togoi*)及斑虻(*Chrysops vittatus*)之磷酸丙糖異構酶(triosephosphate isomerase)有序列相似性 ; 而 35kDa 及 36kDa 過敏原與大葉蟬(*Antheraea yamamai*)及日本橡樹絲蛾(*Homalodisca coagulata*)之果糖 1,6 二磷酸醛縮酶(fructose 1, 6-bisphosphate aldolase)有序列相似性，64kDa 過敏原則與美國龍蝦(*Homarus americanus*)之慢肌肌凝球蛋白之 S1 重鍊(slow muscle myosin S_1 heavy chain)有序列相似性，如表五所示。35kDa 及 36kDa 之蛋白質可能為過敏原同種型(isoform)。根據 WHO/IUIS 之命名原則， 24-、35-/36-及 64kDa 蛋白質依分子量大小暫時命名為 For t 1、For t 2、 及 For t 3， 為台灣鋏蠓之主要過敏原。

22 kDa 蛋白質因含量過微，一直無法取得足量之蛋白質供質譜儀分析，目前尚未知其特性為何。

5.4 討論

吸血蠓之過敏原，不管是在馬[91-93]、羊[94]、牛及驢[95]之「季節性過敏性皮膚炎」或是人類，因研究材料取得的困難性，文獻上除 2005 年我們首先根據本章之研究結果發表台灣鋏蠓之主要過敏原為 For t 1、For t 2 與 For t 3 以外[90]，直到 2009 年 Langner 等人才又報告引起馬季節性過敏性皮膚炎之北美庫蠓(*Culicoides sonorensis*)主要過敏原為 Cul s 1 [127]。

由本章之研究結果，知台灣鋏蠓過敏原 24 kDa 之過敏原與磷酸丙糖異構酶(triosephosphate isomerase)有分子序列之相似性。磷酸丙糖異構酶是一糖解腶，負責催化同分異構物 dihydroxyacetone phosphate 成為 D-glyceraldehyde 3-phosphate。磷酸丙糖異構酶曾被報告為小麥麵粉[128]與乳膠[129;130]之過敏原。

台灣鋏蠓過敏原 35-/36 Da 之過敏原與果糖 1.6 二磷酸醛縮酶有分子序列之相似性。果糖 1.6 二磷酸醛縮酶(fructose 1, 6-bisphosphate aldolase)責司催化 glyceraldehydes 3-phosphate 與 dihydroxyacetone phosphate 之丁間醇醛 (aldol)基使之轉為果糖 1.6 二磷酸(fructose 1, 6-bisphosphate)。在動物與高等植物之第一類醛縮酶(class I aldolase)其催化作用是依賴與其受質之 Schiff base formation[131]，而真菌與原

核生物之第二類醛縮酶(class II aldolase)則需二價陽離子來維持其活性[132]。 利用胺基酸序列分析，37kDa 之醛縮酶曾被報告為引起麵包師傅職業氣喘(baker's asthma)之小麥麵粉過敏原[133;134]。

台灣鋏蠓過敏原 64 kDa 之過敏原與美國龍蝦(*Homarus americanus*)之慢肌肌凝球蛋白之 S1 重鍊(slow muscle myosin S_1 heavy chain)有分子序列之相似性。甲殼類動物之肌肉纖維分為快抽動(fast-twich)型、慢抽動(slow-twich，S1) 型與慢強直(slow-tonic，S2)型[135]。肌凝蛋白(myosin)之重鍊與輕鍊、原肌凝蛋白(tropomyosin，亦稱肌動蛋白)、副肌凝蛋白(paramyosin)、肌鈣蛋白-I (troponin-I)、肌鈣蛋白-T (troponin-T)與肌鈣蛋白-C (troponin-C)在龍蝦與淡水螯蝦(crayfish)中均有被發現具肌纖維專一性同種型[136]。Medler 等人曾報告美國龍蝦之慢抽動 S1 肌凝蛋白重鍊可與肌鈣蛋白-T 之抗體相結合，[137] 且與非洲瘧蚊(Africa malaria mosquito)之某種不明功能之蛋白質有 66%之分子序列相似性。肌鈣蛋白-T 是肌鈣蛋白複合體之一員，與原肌凝蛋白結合後職司鈣離子結合之調控以調節動物之肌肉收縮力。原肌凝蛋白已知是甲殼類、家塵蟎與蟑螂等無脊椎動物之重要泛過敏原[138-141]。我們的研究結果台灣鋏蠓主要過敏原 For t 3 與慢肌肌凝蛋白之 S1 重鍊(slow muscle myosin S_1 heavy chain)有分子序列之相似性，可能也是屬於此一肌纖維蛋白家族之泛過敏原。

總結來說，我們於本章之研究結果找出台灣鋏蠓至少有 11 種過敏原，22-、24-、 35/36- 與 64-kDa 之蛋白質為主要之過敏原，且以蛋白質體學之方法鑑定三個主要過敏原 24kDa，35-/36 kDa 與 64 kDa 之蛋白質身份。

第六章 台灣鋏蠓主要過敏原 For t 1 之分子選殖

6.1 引言

雖然小黑蚊叮咬過敏往往造成患者生活品質之極大困擾，目前卻沒有標準的檢驗方法可供確立診斷，除了請病患僅量避免被叮之外，也沒有積極有效之治療方式。承第五章之研究結果，我們的研究希望能選殖台灣鋏蠓主要過敏原之選株以得到純化之重組過敏原。

目前臨床上診斷免疫球蛋白 E 媒介之過敏病，主要是靠有經驗的臨床醫師依患者之症狀列出可疑過敏原後，再以過敏原皮膚試驗或抽血檢驗病患血液中的過敏原專一型免疫球蛋白 E 來確診。過去一百年來，過敏科醫師均使用天然來源之過敏原粗萃取物作為診斷的工具，而治療過敏病之減敏治療，也是使用粗萃取物作為減敏疫苗。但天然來源之過敏原粗萃取物，常有批次間的變異(batch-to-batch variation)，過敏原蛋白質含量不穩定，含有許多不相關的蛋白質，不容易標準化，有時還有被其他過敏原或細菌內毒素污染的顧慮，有些過敏原使用傳統粗萃取法又不易萃出，使得臨床使用過敏原粗粹物之檢驗試劑，靈敏度與精確度常有不足。而使用過敏原粗粹物之減敏疫苗也有因有效過敏原含量無法精確定量而療效不穩之問題。

6.2 材料與方法

6.2.1 台灣鋏蠓 mRNA 之製備

從野外將新鮮活體之台灣鋏蠓直接凍入-70℃冰箱內。備製 RNA 時先以液態氮將冷凍後的台灣鋏蠓粉末化，隨之加入 TRIzol® 試劑(Invitrogen, CA, USA)，加入氯仿(chloroform)，離心以後，total RNA 會溶於水相層(aqueous phase)。移出水相層溶液，以異丙醇沉澱後，得到 total RNA。

接著利用 Poly(A)Purist™ MAG mRNA 純化套組(Ambion, Austin, TX, USA) 純化 poly A^+ mRNA。Total RNA 於稀釋溶液中與 binding solution 結合，並添加 Oligo(dT)磁珠，使含有 poly(A)序列之 mRNAs 結合上 Oligo(dT)磁珠，兩者相互雜交，將含有磁珠的管子放在磁架上，磁珠就會被吸引至管子的一邊使含有未結合物質及 ribosomal RNA 之上清液輕易的被移除。最後使用預熱好的 RNA Storage Solution 將 poly(A) RNA 洗下來。Poly(A) RNA 從 Oligo(dT) 磁珠洗提後以乙醇沉澱, 定量並立即冷凍至-70℃。

6.2.2 蛋白質體外轉譯(Protein in-vitro translation)

台灣鈸蠓 mRNA 以 biotin 標定之兔子網狀紅血球細胞 Biotin in vitro translation 套組(Roche Diagnostic GmbH, Mannheim, Germany)，進行體外轉譯作用，以確認 mRNA 之活性。這個方法使用 biotin 標定在 lysine-tRNA，轉譯作用合成有 biotin 標定的蛋白質，經 SDS-PAGE 分離後轉移在 PVDF 膜上。利用 streptavidin-peroxidase 抗體來偵測合成的蛋白質，用 luminol/iodphenol 做受質，冷光訊號壓片後呈現在 X 光底片上。

6.2.3 構築台灣鈸蠓 cDNA 基因庫

以 5μg 的台灣鈸蠓 mRNA 為模板，根據 Strategene ZAP-cDNA 合成套組(Stratagene, La Jolla, CA, USA)建議之標準步驟，合成第一股 cytosine 上帶甲基之 cDNA 與 RNA 雜合體。其 Oligo(dT)引子在 5’端便已有一 Xho I 切位，第二股 cDNA 合成後，加入 EcoRI adapters 及 T4 DNA 黏合酶，再以限制酶 Xho I 將 5’端之 Xho I 核甘酸序段切除，使合成之 cDNA 成為一具單向性之雙股 DNA。 再將此雙股 cDNA 產物與入 ZAP XP 載體及 T4 DNA 黏合酶於 4℃下培養 2 天使之黏合入載體，再以 Gigapack III 封裝套組(Stratagene,La Jolla, CA, USA)將含

台灣鋏蠓 cDNA 之λZAP XP 載體封裝入嗜菌體外套，並保存於 SM (0.1M NaCl; 10mM $MgSO_4$; 50mM Tris-HCL, pH 7.5; 0.01% gelatin)緩衝液中，完成台灣鋏蠓 cDNA 基因庫的構築。

6.2.4 以病患血清做蛋白質 cDNA 基因庫篩檢

首先在 42℃將 XL1-Blue MRF'大腸桿菌及λ嗜菌體共同培養 5 小時後，以浸泡過 10mM IPTG(Isopropyl-ß-D-thiogalactopyranosid，Sigma, San Louis, USA)的硝化纖維膜覆蓋，轉至 37℃繼續培養至隔夜，完成誘發λ嗜菌體重組株之融合蛋白的表現。在培養皿和硝化纖維膜做 3 點標記後，小心取出，先以 TBST 緩衝液清洗 30 分鐘後，利用台灣蛺蠓過敏原病人血清(含台灣鋏蠓 IgE 抗體)，進行免疫篩檢(步驟如同前述免疫轉漬法)。呈 IgE 陽性反應之溶菌斑被分離，並再次培養及篩選確認。可能之單一溶班菌株的 cDNA 的片段由兩條λ專一性引子 T3 引子：AATTAACCCTCACTAAAGGGC 和 T7 引子：GTAATACGACTCACTATAGGGC，進行 PCR 增幅。經 99℃加溫 5 分鐘後使雙股 cDNA 變性(denature)，隨之進行 PCR 反應。反應條件如下：94℃，1 分鐘；55℃，1 分鐘；72℃，2 分鐘，共 35 次循環。接著進行 72℃，6 分鐘，最後保存在 4℃。以 1.5%洋菜膠進行 PCR 產物之電泳分析。

6.2.5 過敏原基因定序與同源性序列分析

PCR 之產物經 Taq 聚合酶反應後，會在其產物 3’端加一個 deoxyadenosine(A)。TA cloning 套組(Invitrogen, CA, USA)之 pCR 2.1 載體在 3’端有一 deoxyadenosine(T)，可將 PCR 產物有效地接入載體中。我們使用 TA cloning 方式將 PCR 產物之標的 DNA 接入 pCR 2.1 載體，並轉形（transformation）至大腸桿菌株 Top 10 F, 再以 40μl 40mg/mL 之 X-gal 塗佈於 LB 培養皿中，於 37℃下培養隔夜。由培養箱取出後，於 4℃下 2-3 小時待菌斑呈藍或白色。挑出白色有 DNA 嵌入之單株菌斑做次選殖（subclone）。次選殖後之單株菌斑由台北波仕特生物科技公司做 DNA 定序確認。DNA 與蛋白質序列以 NCBI blastn suite 及 NCBI blastp suite 程式 (http://blast.ncbi.nlm.nih.gov/Blast.cgi)做同源性比對，訊號胜肽(signal peptide)與可能之醣化點則分別使用 ExPASy Proteomics 伺服器（http://www.expasy.ch/）之 NetNGlyc 1.0 程式（http://www.cbs.dtu.dk/services/NetGlycate/）進行分析。

確認無誤之選株抽出質體 DNA 後做進一步次選殖。

6.2.6 以大腸桿菌表現重組過敏原

以限制酶 EcoRI 和 XhoI (Promega)切割選殖得到的過敏原 λ 重組株 cDNA 片段後黏合到 pET-30 表現載體系統(Novagen, Inc., Darmstadt Germany)，轉形至大腸桿菌 BL21 (DE3)菌株 (Stratagen, La Jolla, CA, USA)中。重組株接種在含 25μg/ml kanamycin 的 LB 培養基，在 37℃生長至 OD_{600} 達 0.6~1.0，加入 0.1mM IPTG (Gerbu, Gaiberg, Germany)誘導融合蛋白質的表現。收集經隔夜陪養的菌液，經 8,000g 離心 10 分鐘，倒去培養基，加入 SDS sample buffer 煮沸 5 分鐘後，以 Laemmli 法[124]經 SDS-PAGE(上層 4%，下層 12%)電泳分析融合蛋白質的表現，同時以如 5.2.3 所述之法進行免疫轉漬法，以病人血清確認重組過敏原蛋白質的活性與分子量。

6.2.7 純化重組過敏原

質體 pET30 之融合蛋白質產物的 N 端和 C 端都帶有 6 個組胺酸 histidine residues(稱為 His-tag sequences)，可以結合到二價鎳(Ni^{2+})金屬螯合樹酯的特性加以純化。含過敏原基因的 pET30 重組株經適當的培養和 IPTG 誘導融合蛋白質的產生，收集菌液並根據 Novagen 試劑套組之建議方法進行純化。純化的融合蛋白質利用專一性的

enterokinase (Novagen)在 20℃反應 16 小時，將位於 N 端的載體片段切除(約 5kd)後，得到完整的重組過敏原。

6.2.8 以酵素聯結免疫吸附法(ELISA)分析重組過敏原之 IgE 活性

我們將純化之台灣鋏蠓重組過敏原 3μg/well 以 pH9.6 之鹼性 coating buffer 被覆於 96 孔盤上，於 37℃ 孵育兩小時。以下每步驟之間均以 washing buffer 經過 3 次 washings。Washings 後以 1% 山羊血清(Sigma, San Louis, USA)於室溫 blocking 兩小時後，以 1:10 稀釋之受試者血清為初級抗體，於室溫反應 2 小時。Washings 後再以 mouse anti-human- IgE (BD Pharmigen, San Diego,CA,USA)於室溫下反應兩小時後, 加入 substrate ρ-nitrophenyl phosphate (Sigma, St. Lous,MO, USA)。終止反應後以 Microplate Absorbance Reader (TECAN，Austria)於析光度 405nm 下讀取光學密度值(optic density, O.D.)。

判定為陽性之 cutoff 值定義為 30 名無台灣鋏蠓過敏者反應 O.D. 平均值+2 個標準差(standard deviation)。

6.3 結果

6.3.1 台灣鋏蠓過敏原 cDNA 庫之選殖

在進行台灣鋏蠓過敏原 cDNA 庫之選殖前，先以體外轉譯系統確認台灣鋏蠓訊息核糖核酸(mRNA)純化之品質。台灣鋏蠓訊息核糖核酸經體外轉譯作用之蛋白質產物以 SDS-PAGE 膠電泳分析其結果，如圖十四所示。

構築台灣鋏蠓 cDNA 基因庫前以 ethidium bromide plate assay 半定量所得之 cDNA 產物，約 200ng/μl，如圖十五所示。

取 5μg 的台灣鋏蠓 mRNA 為模板合成雙股 cDNA，以 EcoRI 和 XhoI 切位接入λ ZAP XP 載體構築基因庫後利用台灣鋏蠓過敏病患血清中之 IgE 抗體進行免疫篩選，初次篩選與病患血清反應有陽性冷光訊號的選株經分離後會做次選殖以病患血清反應再次做免疫篩選以二次確認，如圖十六所示。

陽性選株之以 T3 及 T7 引子將 IgE binding clone 做 PCR 增幅，如圖十七所示。

6.3.2 過敏原 TF28-3 之核苷酸序列與胺基酸序列

其中編號 TF28-3 之選株經核苷酸定序，共有 898 個核苷酸，為一 partial clone，其 open reading frame 有 357 個核苷酸，經轉譯後有 118 個胺基酸，為一個 partial clone，如圖十八所示。其換算預估之蛋白質之分子量為 13.67kDa，等電點為 4.84。經 NCBI blastp 比對結果，此蛋白質與埃及斑蚊之 serine/threonine protein kinase 有 65.3%之序列相似性，如圖 19 所示。經向 WHO/IUIS 登錄，選株 TF28-3 正式命名為 For t 1.0101 (Accession number 1088089 ； EU678970)[142] 。

6.3.3 重組過敏原 rFor t 1.0101 與天然台灣鋏蠓過敏原之比較

我們將大腸桿菌表現系統所得之融合重組過敏原 rFor t 1.0101 與台灣鋏蠓粗粹物經 SDS-PAGE 與以病患血清做免疫轉漬法後，在 24kDa 附近有一之明顯之反應，如圖二十所示。

6.3.4 以酵素聯結免疫吸附法(ELISA)測定 rFor t 1.0101 之 IgE 活性

我們將 30 名受試者之血清，其中 28 位為台灣鋏蠓過敏者，兩為為無台灣鋏蠓過敏之對照組以 ELISA 與 rFor t 1.0101 做反應，發現 28 名台灣鋏蠓過敏者中，19 名病患(67.8%)之血清與 rFor t 1.0101 反

應之 O.D.值在 cutoff value 以上，呈陽性反應， 符合主要過敏原之定義，如圖二十一所示。

6.4 討論

我們以台灣鋏蠓 mRNA 構築 cDNA 基因庫，選殖出 For t 1.0101 之部份 cDNA 選株，共有 898 個核苷酸，其 open reading frame 有 357 個核苷酸，經轉譯後有 118 個胺基酸。此蛋白質與埃及斑蚊之絲胺酸/酥胺酸蛋白激脢(serine/threonine protein kinase) 有 65.3%之序列相似性。

蛋白激脢(protein kinase)是一個很大的酵素家族，負責將三磷酸腺苷 (ATP)上之磷酸根移到有氫氧根的胺基酸上做共價結合，使標的蛋白磷酸化而改變其活性與功能，是細胞訊息傳導、功能調節、分裂與分化之重要蛋白質。除動物以外，植物與原核生物之細菌也有蛋白激脢。人類的基因中約有 518 個蛋白激脢之基因，佔人類總基因之 5%[143]。不同生物間的蛋白激脢之催化區(catalytic domain)相當守恆(conserved)，且有許多序列位置已經被找出[144;145]。在蛋白激脢催化區之 N 端，一般含有一富含甘胺酸(glycine-rich)之共同序列 Gly-X-Gly-X-X-Gly，與 ATP 之結合有關[146]。蛋白激脢根據其基質(substrate)專一性之不同來分類，其中最主要的兩類為絲胺酸/酥胺酸專一型(serine/threonine-specific)與酪胺酸專一型(tyrosin-specific)蛋白激脢，其他還有組胺酸專一型(histidine-specific)、天門冬胺酸/

穀胺酸專一型(aspartic acid/glutamic acid-specific) 與混合型等[147-149]。在蛋白激脢催化區之中段如果有 Asp-Leu-Lys-Pro-Glu-Asn 序列則強烈顯示其為一絲胺酸/酥胺酸蛋白激脢；若中段具有 Asp-Leu-Arg-Ala-Ala-Asn(如脊椎動物之 Src 亞科蛋白激脢)或 Asp-Leu-Ala-Ala-Arg-Asn，則為酪胺酸蛋白激脢(tyrosin protein kinase) [150]。

絲胺酸/酥胺酸蛋白激脢(Serine/threonine protein kinase)主司將 serine 或 threonine 上之氫氧根(OH group)磷酸化。細胞訊息傳導重要之蛋白激脢如 protein kinase C、MAP kinases、Mos/Raf kinases 等均為絲胺酸/酥胺酸蛋白激脢的一種。Serine/threonine kinase 在許多種癌細胞均有表現改變(多半是過度表現)之情形[151;152]，也因此成為治療癌症新藥物開發的重要抑制標的[153]。

目前文獻上有報告精胺酸激脢(arginine kinase)為塵蟎之第 20 群(Group 20)過敏原[15]，且亦為美國蟑螂、蝦及蛾之過敏原[154-156]，是無脊椎動物泛過敏原(panallergen)之一[157]，但目前尚未有報告 Serine/threonine protein kinase 為過敏原之文獻。

總結來說，我們在台灣鋏蠓 cDNA 基因庫以病患血清選殖出主要過敏原 rFor t 1.0101，其蛋白質序列與 Serine/threonine protein kinase 有高度之相似性，且過去尚未有此類蛋白激脢為過敏原之報

告，為一新建類之過敏原。

第七章 台灣鋏蠓主要過敏原 For t 2 之分子選殖

7.1 引言

重組過敏原在臨床之運用包括三大領域：診斷、治療及研究。過去的重組過敏原研究顯示，重組過敏原可以取代傳統之天然粹取過敏原用來做體外之過敏原抗體檢測、細胞活化測試與體內之過敏原皮膚試驗之可靠抗原的來源[60;62]。其是如果此一天然過敏原極不易取得(如台灣鋏蠓必需靠人體誘集才能採得)，重組過敏原更能提供一穩定之過敏原之供應源。除了混合多種同一來源過敏原之重組過敏原以取代傳統之天然粹取過敏原用來檢驗之外，Valenta 等人提出將同一來源過敏原多個不同之重組過敏原(如白樺樹花粉之 Bet v 1，Bet v 2, Bet v 3，Bet v 4) 根據其分子特性分別予以檢測[158]，再來分析病患之 IgE 反應資料檔案(profile)，稱為「元件解決診斷法」(component-resolved diagnosis)，比起傳統之過敏原檢測更能準確診斷出病患之致敏來源、臨床嚴重度[159-161]及是否可能對其他過敏原產生交叉反應(cross-reaction)[162-165]，且已有研究者將此一診斷概念發展成重組過敏原晶片以供臨床使用[166;167]，並嘗試以 componet-resolved diagnosis 之結果來改進減敏疫苗的配方[168]。

基於以上的原因，我們雖已選殖出 For t 1.0101，仍有必要繼續選殖台灣鋏蠓之主要過敏原，以便將來能有更完整的臨床用途。因使用病患血清一直無法成功選殖台灣鋏蠓其它主要過敏原，我們決定改以第五章由質譜儀蛋白質身份鑑定已知之 36kDa 過敏原(暫命名為 For t 2)之守恆域(conserve domain)胺基酸胜肽序列飼育專一性抗體來篩選台灣鋏蠓 cDNA 基因庫。

7.2 材料與方法

7.2.1 台灣鋏蠓 mRNA 之製備

從野外將新鮮活體之台灣鋏蠓直接凍入-70℃冰箱內。備製 RNA 時先以液態氮將冷凍後的台灣鋏蠓粉末化，隨之加入 TRIzol® 試劑(Invitrogen, CA, USA)，加入氯仿(chloroform)，離心以後， total RNA 會溶於水相層(aqueous phase)。移出水相層溶液，以異丙醇沉澱後，得到 total RNA。

接著利用 Poly(A)Purist™ MAG mRNA 純化套組(Ambion, Austin, TX, USA) 純化 poly A^+ mRNA。Total RNA 於稀釋溶液中與 binding solution 結合，並添加 Oligo(dT)磁珠，使含有 poly(A)序列之 mRNAs 結合上 Oligo(dT)磁珠，兩者相互雜交，將含有磁珠的管子放在磁架上，磁珠就會被吸引至管子的一邊使含有未結合物質及 ribosomal RNA 之上清液輕易的被移除。最後使用預熱好的 RNA Storage Solution 將 poly(A) RNA 洗下來。Poly(A) RNA 從 Oligo(dT) 磁珠洗提後以乙醇沉澱, 定量並立即冷凍至-70℃。

7.2.2 備置 anti-For t 2 專一性抗體

兩隻2.5公斤重的紐西蘭白兔，分20處於背部皮下注射1mg/ml For t 2 conserve domain ^{183}IVPIVEPEVLPDGEHDLDR^{201} 之胜肽加等體積混合完全Freund佐劑 (Difco Laborary, Detroit, MI)。在第一次免疫注射後，每隔三週再追加注射0.5mg/ml台灣鋏蠓粗萃取物及等體積不完全Freund佐劑，連續三次。採集的兔子血清，先經18% sodium sulfate沉澱後，再經14% sodium sulfate沉澱。純化的For t 2專一性多株IgG抗體經20mM Tris-HCl(pH 8.0) 緩衝液於4℃透析48小時後，分裝並保存在-70℃冰箱。

7.2.3 構築台灣鋏蠓 cDNA 基因庫

以 5μg 的台灣鋏蠓 mRNA 為模板，根據 Strategene ZAP-cDNA 合成套組(Stratagene, La Jolla, CA, USA)建議之標準步驟，合成第一股 cytosine 上帶甲基之 cDNA 與 RNA 雜合體。其 Oligo(dT)引子在 5’端便已有一 Xho I 切位，第二股 cDNA 合成後，加入 EcoRI adapters 及 T4 DNA 黏合酶，再以限制酶 Xho I 將 5’端之 Xho I 核甘酸序段切除，使合成之 cDNA 成為一具單向性之雙股 DNA。 再將此雙股 cDNA 產物與 λZAP XP 載體及 T4 DNA 黏合酶於 4℃下培養 2 天使之黏合入

載體，再以 Gigapack III 封裝套組(Stratagene,La Jolla, CA, USA)將含台灣鋏蠓 cDNA 之λZAP XP 載體封裝入嗜菌體外套，並保存於 SM (0.1M NaCl; 10mM $MgSO_4$; 50mM Tris-HCL, pH 7.5; 0.01% gelatin)緩衝液中，完成台灣鋏蠓 cDNA 基因庫的構築。

7.2.4 以 anti-For t 2 專一性抗體做蛋白質 cDNA 基因庫篩檢

首先在 42℃將 XL1-Blue MRF'大腸桿菌及λ嗜菌體共同培養 5 小時後，以浸泡過 10mM IPTG(Isopropyl-ß-D-thiogalactopyranosid，Sigma, San Louis, USA)的硝化纖維膜覆蓋，轉至 37℃繼續培養至隔夜，完成誘發λ嗜菌體重組株之融合蛋白的表現。在培養皿和硝化纖維膜做 3 點標記後，小心取出，先以 TBST 緩衝液清洗 30 分鐘後，利用 anti-For t 2 抗體進行免疫篩檢(步驟如同前述免疫轉漬法)。呈 IgG 陽性反應之溶菌斑被分離，並再經次培養及篩選確認。可能之單一溶班菌株的 cDNA 的片段由兩條λ專一性引子 T3 引子：AATTAACCCTCACTAAAGGGC 和 T7 引子：GTAATACGACTCACTATAGGGC，進行 PCR 增幅。經 99℃加溫 5 分鐘後使雙股 cDNA 變性(denature)，隨之進行 PCR 反應。反應條件如下：94℃，1 分鐘；55℃，1 分鐘；72℃，2 分鐘，共 35 次循環。接著進行 72℃，6 分鐘，最後保存在 4℃。以 1.5%洋菜膠進行 PCR

產物之電泳分析。

7.2.5 過敏原基因定序與同源性序列分析

PCR 之產物經 Taq 聚合酶反應後，會在其產物 3’端加一個 deoxyadenosine(A)。TA cloning 套組(Invitrogen, CA, USA)之 pCR 2.1 載體在 3’端有一 deoxyadenosine(T)，可將 PCR 產物有效地接入載體中。我們使用 TA cloning 方式將 PCR 產物之標的 DNA 接入 pCR 2.1 載體，並轉形（transformation）至大腸桿菌株 Top 10 F, 再以 40μl 40mg/mL 之 X-gal 塗佈於 LB 培養皿中，於 37℃下培養隔夜。由培養箱取出後，於 4℃下 2-3 小時使菌斑成藍或白色。挑出白色有 DNA 嵌入之單株菌斑做次選殖（subclone）。次選殖後之單株菌斑由台北波仕特生物科技公司做 DNA 定序確認。DNA 與蛋白質序列以 NCBI blastn suite 及 NCBI blastp suite 程式 (http://blast.ncbi.nlm.nih.gov/Blast.cgi)做同源性比對，訊號胜肽(signal peptide)與可能之醣化點則分別使用 ExPASy Proteomics 伺服器（http://www.expasy.ch/）之 NetNGlyc 1.0 程式（http://www.cbs.dtu.dk/services/NetGlycate/）進行分析。

確認無誤之選株抽出質體 DNA 後做進一步次選殖。

7.2.6 以大腸桿菌表現重組過敏原

以限制酶 BamH I 和 Pst I (Promega)切割選殖得到的過敏原 λ 重組株 cDNA 片段後黏合至 QIAexpressionist 套組(Qiagen, CA, USA)之 pQE30 表現載體，再轉形至大腸桿菌 M15[pREP4]菌株(Qiagen, CA, USA)中。重組株接種在含 25μg/ml kanamycin 的 LB 培養基，在 37℃生長至 OD_{600} 達 0.6~1.0，加入 0.05 mM IPTG (Gerbu, Gaiberg, Germany)誘導非融合重組蛋白質的表現。收集經隔夜陪養的菌液，經 8,000g 離心 10 分鐘，倒去培養基，加入 SDS sample buffer 煮沸 5 分鐘後，以 Laemmli 法[124]經 SDS-PAGE(上層 4%，下層 12%)電泳分析融合蛋白質的表現，同時以如 5.2.3 所述之法進行免疫轉漬法，以病人血清確認重組過敏原蛋白質的活性與分子量。

7.2.7 純化重組過敏原

質體 pQE30 之非融合重組蛋白質產物的 N 端和 C 端都帶有 6 個組胺酸 histidine residues(稱為 His-tag sequences)，可以結合到二價鎳(Ni^{2+})金屬螯合樹酯加以純化。含過敏原基因的 pQE30 重組株經適當的培養和 IPTG 誘導融合蛋白質的產生，收集菌液並根據 Qiagen 試劑套組之建議方法進行純化。

7.2.8 以酵素聯結免疫吸附法(ELISA)分析重組過敏原之 IgE 活性

我們將純化之台灣鋏蠓重組過敏原 3μg/well 以 pH9.6 之鹼性 coating buffer 被覆於 96 孔盤上，於 37℃ 孵育兩小時。以下每步驟之間均以 washing buffer 經過 3 次 washings。Washings 後以 1% 山羊血清(Sigma, San Louis, USA)於室溫 blocking 兩小時後，以 1:10 稀釋之病患血清為初級抗體，於室溫反應 2 小時。Washings 後再以 mouse anti-human- IgE (BD Pharmigen, San Diego,CAS,USA)於室溫下反應兩小時後, 加入 substrate ρ-nitrophenyl phosphate (Sigma, St. Lous,MO, USA)。終止反應後以 Microplate Absorbance Reader (TECAN，Austria) 於析光度 405nm 下讀取光學密度值(optic density, O.D.)。

判定為陽性之 cutoff 值定義為 30 名無台灣鋏蠓過敏者反應 O.D. 平均值+2 個標準差(standard deviation)。

7.3 結果

7.3.1 Anti-For t 2 專一性 IgG 抗體之確認

以 For t 2 conserve domain ^{183}IVPIVEPEVLPDGEHDLDR^{201} 之胜肽於兩隻紐西蘭白兔得到之 IgG 抗體，與台灣鋏蠓粗粹物做免疫轉漬法，證實可辨認 36kDa 之台灣鋏蠓蛋白質，與預期辨認之 36kDa For t 2 過敏原位置相符，如圖二十二所示。

7.3.2 台灣鋏蠓過敏原 cDNA 庫之選殖

取 5μg 的台灣鋏蠓 mRNA 為模板合成雙股 cDNA，以 *Eco*RI 和 *Xho*I 切位接入λ ZAP XP 載體構築基因庫後利用 anti-For t 2 專一性 IgG 抗體進行免疫篩選，初次篩選與 anti-For t 2 抗體有陽性冷光訊號反應的選株經分離後會做次選殖以 anti-For t 2 抗體反應再次做免疫篩選以二次確認。陽性選株之以 T3 及 T7 引子將 IgE-binding clone 做 PCR 增幅，如圖二十三所示。

7.3.3 過敏原 TF210 之核苷酸序列與胺基酸序列

其中編號 TF210 之選株經核苷酸定序，共有 1065 個核苷酸，為一

full clone，15-992 為 coding region，其 open reading frame 有 978 個核苷酸，經轉譯後有 325 個胺基酸，如圖二十四所示。其換算預估之蛋白質之分子量為 36kDa，等電點為 5.28。經 NCBI blastp 比對結果，此蛋白質與埃及斑蚊之真核細胞轉譯起始因子 3 亞單位(eukaryotic translation initiation factor 3 subunit)有 77.9%之序列相似性，如圖二十五所示。經向 WHO/IUIS 登錄，選株 TF210 正式命名為 For t 2.0101(Accession number 1088115； EU678971)[169] 。

7.3.4 以免疫轉漬法測定重組過敏原 rFor t 2.0101 之 IgE 結合活性

因 anti-For t 2 抗體所篩選之選株為一具 IgG 結合活性之選株，我們必須再確認其表現之蛋白質具 IgE 結合活性。我們將大腸桿菌表現系統所得之重組過敏原 rFor t 2.0101 經 SDS-PAGE 電泳後與病患血清做免疫轉漬法後，具有與病患免疫球蛋白 E 結合之能力，如圖二十六所示，確實為一過敏原。

7.3.5 以酵素聯結免疫吸附法(ELISA)測定 rFor t 2.0101 之 IgE 結合活性

我們將 30 名受試者之血清，其中 28 位為台灣鋏蠓過敏者，兩位為無台灣鋏蠓過敏之對照組以 ELISA 與 rFor t 2.0101 做反應，發現

28 名台灣鋏蠓過敏者中，21 名病患(75%)之血清與 rFor t 2.0101 反應之 O.D.值在 cutoff value 以上，呈陽性反應， 符合主要過敏原之定義，如圖二十七所示。

7.4 討論

我們以台灣鋏蠓 mRNA 構築 cDNA 基因庫，選殖出 For t 2.0101 之全長 cDNA ，共有 1065 個核苷酸，15-992 為編碼區(coding region)，其開放讀碼框(open reading frame)有 978 個核苷酸，經轉譯後有 325 個胺基酸，蛋白質之分子量為 36kDa，等電點為 5.28 且與埃及斑蚊之真核細胞轉譯起始因子 3 亞單位(eukaryotic translation initiation factor 3 subunit, eIF3 subunit)有 77.9%之序列相似性。

真核細胞蛋白質轉譯過程經過多重反應步驟之後，最終形成一含甲硫胺醯基起始 tRNA (Methionyl iniator tRNA, Met-tRNA$_i^{MET}$)與 AUG 起始碼及核糖體(ribosome) P site 鹼基配對之 80S 起始複合體。目前已知有多個真核細胞轉譯起始因子參與此一反應。蛋白質轉譯的初期 ，40S 核糖體亞單位會與 eIF3 形成複合體，60S 核糖體亞單位則與 eIF6 形成複合體。接著 eIF1A 與 40S 核糖體亞單位-eIF3 複合體結合再與 Met-tRNA$_i^{MET}$、GTP 及 eIF2 組成之三元複合體結合，成一前起始複合體(preinitiation complex)[170;171]。在蛋白質轉譯開始時，mRAN 之 5’覆蓋(5’cap)會與 eIF4 之 cap-binding complex 結合。這個 mRNA-eIF4 複合體再與前起始複合體上之 eIF3 作用而形成完整的起始複合體(initiation complex)，開始蛋白質轉譯前之掃描以辨識起

始碼核苷酸 AUG[170]。

因為真核細胞轉譯起始因子對真核細胞基因表現在 mRNA 轉譯為蛋白質的層次的調控居極重要的角色，此一步驟的調控異常與細胞生長甚至癌化有很密切的相關。最近的研究發現 eIF3 亞單位在人類癌細胞有表達之改變，且其改變與癌症之生成及預後有關。Asano 等人也發現小鼠的 eIF3 基因 Int-6 為小鼠乳腺腫瘤病毒之嵌入區，有可能為細胞癌變之一重要變異區[172-176]。

目前文獻上尚無 eIF3 為過敏原之報告，如同 For t 1.0101，也是一新建類之過敏原。但 Oh 等人於 2001 年曾發表同為真核細胞轉譯起始因子之 eIF6 mRNA 在交聯(cross-linking)小鼠與人類肥大細胞(mast cells)之 IgE 高結合力受體 FcεRI 後會高度表達且此一表達可被腎上腺皮質醇 dexamethasone 及鈣調磷酸酶抑制劑(calcineurin inhibitor) 環孢靈 A(cyclosporine A)所阻斷。若在肥大細胞內過度表達 eIF6 則肥大細胞之組織胺與第二介白質之產量會增加，顯示真核細胞轉譯起始因子 6 在過敏發炎反應也有其角色[177]。具 eIF3 分子特性之 For t 2.0101 是否也有同樣之免疫刺激作用，需要我們進一步之研究。

第八章 結論與展望

我們的研究，由台灣鋏蠓叮咬過敏的流行病學調查開始，證實有高達 60%之被叮咬者會有程度不同的過敏反應， 且其反應分為立即型過敏反應與延遲型過敏反應兩種型態。我們也發現台灣鋏蠓叮咬立即型過敏反應為一免疫球蛋白 E 媒介之第 I 型過敏反應，台灣鋏蠓叮咬之延遲型過敏反應則牽涉淋巴球之增生、Th1 免疫反應細胞激素丙型干擾素之分增加與先天免疫細胞激素第六介白質與甲型腫瘤壞死因子之分泌，較偏第 IV 型之過敏反應。台灣鋏蠓叮咬之劇癢反應確實為一過敏反應而非其唾液引起之毒性反應。我們也發現蛋白胺活化受體發炎路徑與此台灣鋏蠓劇癢之叮咬過敏反應也有關係。

我們以免疫轉漬法發現台灣鋏蠓至少有 11 個致敏蛋白質可與病患血清反應。 22kDa, 24kDa, 36kDa 及 64kDa 之過敏原可分別超過 50%之病患血清反應，符合世界衛生組織所定義之主要過敏原。我們接著以 λZAPII 嗜菌體建立台灣鋏蠓之 cDNA 庫，並以病患血清或 For t 2 專一性抗體來篩選菌株。我們以病患血清 IgE 或 For t 2 專一性抗體反應之選株次選殖至 pET30 或 pQE30 載體並以 *E. coli* 表現系統表現並純化其表現之重組蛋白質。我們成功表現並純化一 24kDa 融合重組過敏原 rFor t 1.0101 (Accession number1088089；EU678970)

及一 36kDa 重組過敏原 rFor t 2.0101 (Accession number 1088115；EU678971)。 rFor t 1.0101 與 67.8%之病患 IgE 反應而 rFor t 2.0101 與 75%之病患血清反應，均符合主要過敏原之定義。rFor t 1.0101 與埃及斑蚊之 serine/threonine protein kinase 有 65.3%之序列相似性而 rFor t 2.0101 sequence 與埃及斑蚊之 eukaryotic translation initiation factor 3 subunit 有 77.9%之相似性。

我們實驗室目前還繼續進行小黑蚊叮咬與人體蛋白胺活化受體有關之非 IgE 媒介發炎反應之研究。我們也正使用 rFor t 1.0101 及 rFor t 2.0101 重組過敏原刺激人類皮膚角質細胞及纖維細胞，希望能了解這兩個新建類之過敏原對不同先天免疫細胞之免疫活性為何。另一方面，我們也以純化之台灣鋏蠓重組過敏原篩檢更大量的受試者族群，以取得這兩個重組過敏原血清流行病學之資料，做為發展台灣鋏蠓過敏及皮膚試驗試劑之驗證材料並希望這些純化之重組過敏原將來可做為台灣鋏蠓過敏減敏治療疫苗之來源。

九、參考文獻

References

(1) Kay AB. Allergy and Hypersensitivity:History and Concept. In: Kay AB, Kaplan AP, Bousquet J, Holt PG, editors. Allergy and Allergic Diseases. Oxford, UK: Wiley-Blackwell, 2008: 3-22.

(2) von Priquet C. Allergie. Munchen: Med Wehnschr 53, 1906.

(3) Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 2004; 113(5):832-6.

(4) Johansson SG, Hourihane JO, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T et al. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. Allergy 2001; 56(9):813-24.

(5) Ishizaka K, Ishizaka T, Hornbrook MM. Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity wth gamma-E-globulin antibody. J Immunol 1966; 97(6):840-53.

(6) Ishizaka K, Ishizaka T. Identification of gamma-E-antibodies as a carrier of reaginic activity. J Immunol 1967; 99(6):1187-98.

(7) Coombs RRA, Gell PGH. The classification of allergic reactions responsible for allergic reactions underlying diseases. In: Gell PGH, Coombs RRA, editors. Clinical Aspects of Immunology. Oxford: Blackwell Scientific Publications, 1963: 217-37.

(8) Kay AB. Concepts of allergy and hypersensitivity. In: Kay AB, editor. Allergy and Allergic Diseases. Oxford, UK: Blackwell Science, 1997: 23-35.

(9) Hamilton RG. Laboratory Tests for Allergic and Immunodeficiency Diseases: Principles and Interpretations. In: Adkinson NF, Jr., Bochner BS, Busse WW, Holgate ST, Lemanske RFJ, Simons FER, editors. Middleton's Allergy - Principles & Practice. Philadelphia,PA: Mosby Elsevier, 2009: 1247-66.

(10) Bochner BS, Busse WW. Advances in mechanisms of allergy. J Allergy Clin Immunol 2004; 113(5):868-75.

(11) Ushio H. [Toll-like receptor and allergy]. Arerugi 2004; 53(5):469-75.

(12) Chinen J, Shearer WT. Advances in basic and clinical immunology in 2007. J Allergy Clin Immunol 2008; 122(1):36-41.

(13) Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. J Allergy Clin Immunol 2004; 114(5):997-1008.

(14) Sigsgaard T, Hoffmann HJ, Thorne PS. The role of innate immunity in occupational allergy: recent findings. Curr Opin Allergy Clin Immunol 2008; 8(2):120-5.

(15) Stewart GA, Zhang J, Robinson C. The Structure and Function of Allergens. In: Adkinson NF, Jr., Bochner BS, Busse WW, Holgate ST, Lemanske RFJ, Simons FER, editors. Middleton's Allergy - Principles & Practice. Philadelphia,PA: Mosby Elsevier, 2009: 569-608.

(16) Aalberse RC, van RR. Crossreactive carbohydrate determinants. Clin Rev Allergy Immunol 1997; 15(4):375-87.

(17) Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. Clin Exp Allergy 2004; 34(1):137-44.

(18) Chapman MD. Allergen nomenclature. Clin Allergy Immunol 2008; 21:47-58.

(19) Larsen JN, Lowenstein H. Allergen nomenclature. J Allergy Clin Immunol 1996; 97(2):577-8.

(20) Allergen nomenclature. WHO/IUS Allergen Nomenclature Subcommittee World Health Organization, Geneva, Switzerland. Clin Exp Allergy 1995; 25(1):27-37.

(21) Allergen nomenclature. IUIS/WHO Allergen Nomenclature Subcommittee. Bull World Health Organ 1994; 72(5):797-806.

(22) King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TA, Thomas W. Allergen nomenclature. WHO/IUIS Allergen Nomenclature

Subcommittee. Int Arch Allergy Immunol 1994; 105(3):224-33.

(23) International Union of Immunological Societies Allergen Nomenclature Sub-Committee. Allergen Nomenclature. The Official list of allergens . 12-4-2008.
Ref Type: Electronic Citation

(24) Allergen nomenclature. WHO/IUS Allergen Nomenclature Subcommittee World Health Organization, Geneva, Switzerland. Clin Exp Allergy 1995; 25(1):27-37.

(25) Lan JL, Lee DT, Wu CH, Chang CP, Yeh CL. Cockroach hypersensitivity: preliminary study of allergic cockroach asthma in Taiwan. J Allergy Clin Immunol 1988; 82(5 Pt 1):736-40.

(26) Kang BC. Cockroach allergy. Clin Rev Allergy 1990; 8(1):87-98.

(27) Sakaguchi M, Inouye S, Miyazawa H, Okabe T, Yasueda H, Muto A et al. Sensitization to cockroach allergens of asthma patients in Japan. Arerugi 1994; 43(11):1309-15.

(28) Garcia DP, Corbett ML, Sublett JL, Pollard SJ, Meiners JF, Karibo JM et al. Cockroach allergy in Kentucky: a comparison of inner city, suburban, and rural small town populations. Ann Allergy 1994; 72(3):203-8.

(29) Baur X, Liebers V. Insect hemoglobins (Chi tI) of the diptera family Chironomidae are relevant environmental, occupational, and hobby-related allergens. Int Arch Occup Environ Health 1992; 64(3):185-8.

(30) Kino T, Chihara J, Fukuda K, Sasaki Y, Shogaki Y, Oshima S. Allergy to insects in Japan. III. High frequency of IgE antibody responses to insects (moth, butterfly, caddis fly, and chironomid) in patients with bronchial asthma and immunochemical quantitation of the insect-related airborne particles smaller than 10 microns in diameter. J Allergy Clin Immunol 1987; 79(6):857-66.

(31) Levine MI, Lockey RF. Monograph on insect allergy. 4th ed. Milwaukee: American Academy of Allergy Asthma & Immunology, 2003.

(32) Frazier CA. Allergic reactions to biting insects. Lancet 1972; 1(7756):911.

(33) Hoffman DR. Allergic Reactions to Biting Insects. In: Levine MI,

Lockey RF, editors. Monograph on Insect Allergy. Milwaukee: American Academy of Allergy Asthma & Immunology, 2003: 161-74.

(34) Golden DB. Stinging insect allergy. Am Fam Physician 2003; 67(12):2541-6.

(35) Golden DB, Kagey-Sobotka A, Norman PS, Hamilton RG, Lichtenstein LM. Outcomes of allergy to insect stings in children, with and without venom immunotherapy. N Engl J Med 2004; 351(7):668-74.

(36) Reisman RE. Stinging insect allergy. J Allergy Clin Immunol 1979; 64(1):3-4.

(37) Yocum MW, Khan DA. Assessment of patients who have experienced anaphylaxis: a 3-year survey. Mayo Clin Proc 1994; 69(1):16-23.

(38) Mullins RJ. Anaphylaxis: risk factors for recurrence. Clin Exp Allergy 2003; 33(8):1033-40.

(39) Kahan E, Ben-Moshe R, Derazne E, Tamir R. The impact of Hymenoptera venom allergy on occupational activities. Occup Med (Lond) 1997; 47(5):273-6.

(40) Oude Elberink JN, De Monchy JG, Van Der HS, Guyatt GH, Dubois AE. Venom immunotherapy improves health-related quality of life in patients allergic to yellow jacket venom. J Allergy Clin Immunol 2002; 110(1):174-82.

(41) Golden DB. Insect sting allergy and venom immunotherapy. Ann Allergy Asthma Immunol 2006; 96(2 Suppl 1):S16-S21.

(42) Muller U, Berchtold E, Helbling A. Honeybee venom allergy: results of a sting challenge 1 year after stopping successful venom immunotherapy in 86 patients. J Allergy Clin Immunol 1991; 87(3):702-9.

(43) Peng Z, Simons FE. Advances in mosquito allergy. Curr Opin Allergy Clin Immunol 2007; 7(4):350-4.

(44) Peng Z, Yang M, Simons FE. Measurement of mosquito Aedes vexans salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. Ann Allergy Asthma Immunol 1995; 74(3):259-64.

(45) Peng Z, Simons F.E.R. Mosquito Allergy: Immune Mechanisms and

Recombinant Salivary Allergens. Int Arch Allergy Immunol 2004; 133:198-209.

(46) Peng Z, Yang M, Simons FE. Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens. Ann Allergy Asthma Immunol 1996; 77(3):238-44.

(47) Peng Z, Xu W, James AA, Lam H, Sun D, Cheng L et al. Expression, purification, characterization and clinical relevance of rAed a 1--a 68-kDa recombinant mosquito Aedes aegypti salivary allergen. Int Immunol 2001; 13(12):1445-52.

(48) Hoffman DR. Allergens in Hymenoptera venom. XVI: Studies of the structures and cross-reactivities of vespid venom phospholipases. J Allergy Clin Immunol 1986; 78(2):337-43.

(49) Schmidt JO, Blum MS, Overal WL. Comparative enzymology of venoms from stinging Hymenoptera. Toxicon 1986; 24(9):907-21.

(50) Hoffman DR, Sakell RH, Schmidt M. Sol i 1, the phospholipase allergen of imported fire ant venom. J Allergy Clin Immunol 2005; 115(3):611-6.

(51) Peng Z, Li H, Simons FE. Immunoblot analysis of salivary allergens in 10 mosquito species with worldwide distribution and the human IgE responses to these allergens. J Allergy Clin Immunol 1998; 101(4 Pt 1):498-505.

(52) Brummer-Korvenkontio H, Palosuo T, Francois G, Reunala T. Characterization of Aedes communis, Aedes aegypti and Anopheles stephensi mosquito saliva antigens by immunoblotting. Int Arch Allergy Immunol 1997; 112(2):169-74.

(53) Valenzuela JG, Pham VM, Garfield MK, Francischetti IM, Ribeiro JM. Toward a description of the sialome of the adult female mosquito Aedes aegypti. Insect Biochem Mol Biol 2002; 32(9):1101-22.

(54) Peng Z, Xu W, Lam H, Cheng L, James AA, Simons FE. A new recombinant mosquito salivary allergen, rAed a 2: allergenicity, clinical relevance, and cross-reactivity. Allergy 2006; 61(4):485-90.

(55) Li C, Beckett AN, James AA, Peng Z. Diagnosis of mosquito allergy: role of a new 67kDa recombinant Aedes aegypti salivary allergen: rAed a 4. Allergy Clin Immunol Int J World Allergy Org Suppl, 195. 2003.

Ref Type: Abstract

(56) Wongkamchai S, Techasintana P, Wisuthsarewong W, Kulthanan K, Suthipinittharm P, Eakpo P. Analysis of IgE-binding allergens in Culex quinquefasciatus saliva protein in mosquito bite allergic patients. Ann Allergy Asthma Immunol 2007; 98(2):200-1.

(57) Oude Elberink JN, De Monchy JG, Van Der HS, Guyatt GH, Dubois AE. Venom immunotherapy improves health-related quality of life in patients allergic to yellow jacket venom. J Allergy Clin Immunol 2002; 110(1):174-82.

(58) Srivastava D, Singh BP, Sudha VT, Arora N, Gaur SN. Immunotherapy with mosquito (Culex quinquefasciatus) extract: a double-blind, placebo-controlled study. Ann Allergy Asthma Immunol 2007; 99(3):273-80.

(59) Niederberger V, Valenta R. Recombinant allergens for immunotherapy. Where do we stand? Curr Opin Allergy Clin Immunol 2004; 4(6):549-54.

(60) Valenta R. Biochemistry of Allergens and Recombinant Allergens. In: Kay AB, Kaplan AP, Bousquet J, Holt PG2, editors. Allergy and Allergic Diseases. Oxford, UK: Wiley-Blackwell, 2008: 895-912.

(61) Mothes N, Valenta R, Spitzauer S. Allergy testing: the role of recombinant allergens. Clin Chem Lab Med 2006; 44(2):125-32.

(62) Valenta R. Recombinant allergen-based concepts for diagnosis and therapy of type I allergy. Allergy 2002; 57 Suppl 71:66-7.

(63) Valenta R, Kraft D. Recombinant allergen molecules: tools to study effector cell activation. Immunol Rev 2001; 179:119-27.

(64) Ballmer-Weber BK, Wangorsch A, Bohle B, Kaul S, Kundig T, Fotisch K et al. Component-resolved in vitro diagnosis in carrot allergy: does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure? Clin Exp Allergy 2005; 35(7):970-8.

(65) Cromwell O, Suck R, Kahlert H, Nandy A, Weber B, Fiebig H. Transition of recombinant allergens from bench to clinical application. Methods 2004; 32(3):300-12.

(66) Moingeon P. Sublingual immunotherapy: from biological extracts to recombinant allergens. Allergy 2006; 61 Suppl 81:15-9.

(67) Valenta R, Niederberger V. Recombinant allergens for immunotherapy. J Allergy Clin Immunol 2007; 119(4):826-30.

(68) 連日清. 小黑蚊 (銳蠓屬蠛蠓亞屬) 之分類、生態及防治. 1-7. 2008. 小黑蚊之發生、生態及防治研討會專刊.
Ref Type: Serial (Book, Monograph)

(69) Sun W. Study of a biting midge, Forcipomyia(Lasiohelea) taiwana (Shiraki)(Diptera: Ceratopogonidae)I.Description of the complete life cycle of the midge reared in the laboratory. Bio Bull Tunghai Univ (Taiwan) 29, 1-10. 1967.
Ref Type: Journal (Full)

(70) Yeh CC, Chuang YY. Colonization and Bionomics of Forcipomyia taiwana (Diptera: Ceratopog*onidae) in* l*aborato*ry. J Med Entomol , 445-8. 1996.
Ref Type: Journal (Full)

(71) 陳錦生. 花蓮地區台灣銳蠓之形態及族群動態研究. 國立臺灣大學/植物病蟲害研究所, 1980.

(72) Shiraki T. Investigation on general injurious insect. Taiwan Sotokufu Noji Shikenjo Tokubetsu Hokodu 8, 286-97. 1913.
Ref Type: Journal (Full)

(73) Chuang YY, Lin CS, Wang CH, Yeh CC. Distribution and seasonal occurrence of Forcipomyia taiwana (Diptera: Ceratopogonidae) in the Nantou area in Taiwan. J Med Entomol 2000; 37(2):205-9.

(74) Chen CS, Lien JC, Lin YN, Hsu SJ. The diurnal biting pattern of a bloodsucking midge Forcipomyia (Lasiohelea) taiwana (Shiraki) (Diptera, Ceratopogonidae). Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 1981; 14(1):54-6.

(75) 莊益源. 台灣銳蠓之生活史及其在南投地區之季節消長. 國立中興大學/昆蟲學系, 1994.

(76) Chen C, Lin Y, Chung C, Hung H. Preliminary observation on the larval breeding sites and adult resting places of a bloodsucking midge, Forcipomyia(lasiohelea) taiwana(Shiraki) (Diptera:Ceratopogonidae). Bull Soc Entomol Natl Chung-Hsing Univ Taiwan 14, 51-9. 1979.
Ref Type: Journal (Full)

(77) 羅偉誠. 臺灣銳蠓攜帶病原體潛在性與吸血源之研究. 中台科技大學生命科學研究所, 2008.

(78) 王惠鵬. 南投地區台灣銳蠓之化學防治. 國立中興大學昆蟲學系, 1996.

(79) 張伯熙. 台灣銳蠓之殺蟲劑篩選及其誘集研究. 國立中興大學昆蟲學系, 1996.

(80) 鄧勝軒. 坡地社區台灣銳蠓防治之研究. 國立中興大學水土保持學系, 2004.

(81) 陳佩琇. 食物及溼度對台灣銳蠓 (雙翅目：蠓科) 發育之影響. 國立中興大學昆蟲學系, 2005.

(82) Yunginger JW. History of Hymenotera Allergy. In: Levine MI, Lockey RF, editors. Monograph on Insect Allergy. Milwaukee, WI: American Academy of Allergy, Asthma and Immunology, 2003: 1-10.

(83) Cohen SG, Samter M. Excepts From Classics in Allergy. 2nd ed. Carlsbad, CA: Symposia Foundation, 1993.

(84) Muller UR. Insect Sting Allergy. In: Kay AB, Kaplan AP, Bousquet J, Holt PG2, editors. Allergy and Allergic Diseases. Oxford, UK: Wiley-Blackwell, 2008: 1980-94.

(85) 李學進. 台灣小黑蚊研究之過去與未來. 1-15. 2008. 小黑蚊之發生、生態及防治研討會專刊.
Ref Type: Serial (Book,Monograph)

(86) 黃杏英 . 1.台灣蛺蠓形態之研究 2.台灣蛺蠓叮咬後之血清病學研究. 中山醫學院生物化學研究所, 2000.

(87) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.

（88） Norman PS, Lichtenstein LM, Ishizaka K. Diagnostic tests in ragweed hay fever. A comparison of direct skin tests, IgE antibody measurements, and basophil histamine release. J Allergy Clin Immunol 1973; 52(4):210-24.

(89) Alsenz J, Loos M. A rapid and simple ELISA for the determination of duplicate monoclonal antibodies during epitope analysis of antigens and its application to the study of C1(-)-INH. J Immunol Methods 1988; 109(1):75-84.

(90) Chen YH, Lee MF, Lan JL, Chen CS, Wang HL, Hwang GY et al. Hypersensitivity to Forcipomyia taiwana (biting midge): clinical analysis and

identification of major For t 1, For t 2 and For t 3 allergens. Allergy 2005; 60(12):1518-23.

(91) Hellberg W, Wilson AD, Mellor P, Doherr MG, Torsteinsdottir S, Zurbriggen A et al. Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to Culicoides nubeculosus salivary gland extract. Vet Immunol Immunopathol 2006; 113(1-2):99-112.

(92) Wilson AD, Harwood LJ, Bjornsdottir S, Marti E, Day MJ. Detection of IgG and IgE serum antibodies to Culicoides salivary gland antigens in horses with insect dermal hypersensitivity (sweet itch). Equine Vet J 2001; 33(7):707-13.

(93) Anderson GS, Belton P, Kleider N. Hypersensitivity of horses in British Columbia to extracts of native and exotic species of Culicoides (Diptera: Ceratopogonidae). J Med Entomol 1993; 30(4):657-63.

(94) Yeruham I, Perl S, Braverman Y. Seasonal allergic dermatitis in sheep associated with Ctenocephalides and Culicoides bites. Vet Dermatol 2004; 15(6):377-80.

(95) Yeruham I, Braverman Y, Orgad U. Field observations in Israel on hypersensitivity in cattle, sheep and donkeys caused by Culicoides. Aust Vet J 1993; 70(9):348-52.

(96) Chen YL, Simons FE, Peng Z. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. Int Arch Allergy Immunol 1998; 116(4):269-77.

(97) Billingsley PF, Baird J, Mitchell JA, Drakeley C. Immune interactions between mosquitoes and their hosts. Parasite Immunol 2006; 28(4):143-53.

(98) Peng Z, Beckett AN, Engler RJ, Hoffman DR, Ott NL, Simons FE. Immune responses to mosquito saliva in 14 individuals with acute systemic allergic reactions to mosquito bites. J Allergy Clin Immunol 2004; 114(5):1189-94.

(99) Lymphocyte function. In: Hay FC, Westwood OMR, editors. Practical Immunology. Oxford: Blackwell Science, 2002: 261-300.

(100) Kellar KL, Kalwar RR, Dubois KA, Crouse D, Chafin WD, Kane BE. Multiplexed fluorescent bead-based immunoassays for quantitation of human cytokines in serum and culture supernatants. Cytometry 2001; 45(1):27-36.

(101) Yang DL, Kraght P, Pentoney CS, Pentoney SL, Jr. Analytical significance of encroachment in multiplexed bead-based flow cytometric assays. Anal Chem 2007; 79(10):3607-14.

(102) Chavira R, Jr., Burnett TJ, Hageman JH. Assaying proteinases with azocoll. Anal Biochem 1984; 136(2):446-50.

(103) Page K, Hughes VS, Odoms KK, Dunsmore KE, Hershenson MB. German cockroach proteases regulate interleukin-8 expression via nuclear factor for interleukin-6 in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2005; 32(3):225-31.

(104) Page K, Strunk VS, Hershenson MB. Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. J Allergy Clin Immunol 2003; 112(6):1112-8.

(105) Peng Z, Yang M, Simons FE. Measurement of mosquito Aedes vexans salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. Ann Allergy Asthma Immunol 1995; 74(3):259-64.

(106) Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001; 104(4):487-501.

(107) Doganci A, Sauer K, Karwot R, Finotto S. Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. Clin Rev Allergy Immunol 2005; 28(3):257-70.

(108) Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH et al. Evidence of a role of tumor necrosis factor alpha in refractory asthma. N Engl J Med 2006; 354(7):697-708.

(109) Carballido JM, Carballido-Perrig N, Terres G, Heusser CH, Blaser K. Bee venom phospholipase A2-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to the antigen concentration. Eur J Immunol 1992; 22(6):1357-63.

(110) Levi-Schaffer F, Temkin V, Malamud V, Feld S, Zilberman Y. Mast cells enhance eosinophil survival in vitro: role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. J Immunol 1998; 160(11):5554-62.

(111) Akdis CA, Joss A, Akdis M, Blaser K. Mechanism of IL-10-induced T

cell inactivation in allergic inflammation and normal response to allergens. Int Arch Allergy Immunol 2001; 124:180-2.

(112) Jutel M, Akdis M, Budak F, ebischer-Casaulta C, Wrzyszcz M, Blaser K et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003; 33(5):1205-14.

(113) Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. J Exp Med 2008; 205(12):2887-98.

(114) Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006; 6(10):761-71.

(115) Landis RC. Protease activated receptors: clinical relevance to hemostasis and inflammation. Hematol Oncol Clin North Am 2007; 21(1):103-13.

(116) Hong JH, Lee SI, Kim KE, Yong TS, Seo JT, Sohn MH et al. German cockroach extract activates protease-activated receptor 2 in human airway epithelial cells. J Allergy Clin Immunol 2004; 113(2):315-9.

(117) Kauffman HF, Tamm M, Timmerman JA, Borger P. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. Clin Mol Allergy 2006; 4:5.

(118) Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. J Allergy Clin Immunol 2000; 105(6 Pt1):1185-93.

(119) Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS et al. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 2003; 23(15):6176-80.

(120) Babiarz-Magee L, Chen N, Seiberg M, Lin CB. The expression and activation of protease-activated receptor-2 correlate with skin color. Pigment Cell Res 2004; 17(3):241-51.

(121) Steinhoff M, Corvera CU, Thoma MS, Kong W, McAlpine BE, Caughey GH et al. Proteinase-activated receptor-2 in human skin: tissue distribution

and activation of keratinocytes by mast cell tryptase. Exp Dermatol 1999; 8(4):282-94.

(122) Adkinson NFA, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons FER. Middletons's Allergy:Principles & Practice. 6th ed. Philadelphia: Mosby, Inc., 2003.

(123) Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. Clin Exp Allergy 2004; 34(1):137-44.

(124) Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 19770; 252(3):1102-6.

(125) Towbin H, Staehelin T, Gordon J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Nati Acad Sci USA 1979; 76:4350-4.

(126) Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999; 20(18):3551-67.

(127) Langner KF, Jarvis DL, Nimtz M, Heselhaus JE, McHolland LE, Leibold W et al. Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge Culicoides sonorensis relevant for summer eczema in horses. Int J Parasitol 2009; 39(2):243-50.

(128) Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. J Allergy Clin Immunol 2001; 107(5):907-13.

(129) Posch A, Chen Z, Wheeler C, Dunn MJ, Raulf-Heimsoth M, Baur X. Characterization and identification of latex allergens by two-dimensional electrophoresis and protein microsequencing. J Allergy Clin Immunol 1997; 99(3):385-95.

(130) Posch A, Chen Z, Dunn MJ, Wheeler CH, Petersen A, Leubner-Metzger G et al. Latex allergen database. Electrophoresis 1997; 18(15):2803-10.

(131) Grazi E, Rowley PT, Cheng T, Tchola O, Horecker BL. The

mechanism of action of aldolases. III. Schiff base formation with lysine. Biochem Biophys Res Commun 1962; 9:38-43.

(132) Rutter WJ. Evolution of Aldolse. Fed Proc 1964; 23:1248-57.

(133) Weiss W, Huber G, Engel KH, Pethran A, Dunn MJ, Gooley AA et al. Identification and characterization of wheat grain albumin/globulin allergens. Electrophoresis 1997; 18(5):826-33.

(134) Baur X, Posch A. Characterized allergens causing bakers' asthma. Allergy 1998; 53(6):562-6.

(135) Mykles DL. Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. Comp Biochem Physiol B Biochem Mol Biol 1997; 117(3):367-78.

(136) Mykles DL, Medler S, Koenders A, Cooper R. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J Exp Biol 2002; 205(Pt 4):513-22.

(137) Medler S, Mykles DL. Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster Homarus americanus: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres. J Exp Biol 2003; 206(Pt 20):3557-67.

(138) Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch Allergy Immunol 1999; 119(4):247-58.

(139) Leung PS, Chen YC, Gershwin ME, Wong SH, Kwan HS, Chu KH. Identification and molecular characterization of Charybdis feriatus tropomyosin, the major crab allergen. J Allergy Clin Immunol 1998; 102(5):847-52.

(140) Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (Penaeus aztecus) allergen as the muscle protein tropomyosin. Int Arch Allergy Immunol 1994; 105(1):49-55.

(141) Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. J Immunol 1993; 151(10):5354-63.

(142) Chen Y-H, LMF, CSS, Hwang G-Y. EU678970 Forcipomyia taiwana serine/threonine protein kinase mRNA, partial cds. http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=189030777 . 2008.

(143) Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002; 298(5600):1912-34.

(144) Hanks SK. Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. Genome Biol 2003; 4(5):111.

(145) Hanks SK, Quinn AM. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol 1991; 200:38-62.

(146) Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 1988; 241(4861):42-52.

(147) Hunter T. A thousand and one protein kinases. Cell 1987; 50(6):823-9.

(148) Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J 1995; 9(8):576-96.

(149) Hunter T. Protein kinase classification. Methods Enzymol 1991; 200:3-37.

(150) Taylor SS, Radzio-Andzelm E, Hunter T. How do protein kinases discriminate between serine/threonine and tyrosine: Structural insights from the insulin receptor protein-tyrosine kinase. FASEB J 1995; 9(13):1255-66.

(151) Capra M, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M et al. Frequent alterations in the expression of serine/threonine kinases in human cancers. Cancer Res 2006; 66(16):8147-54.

(152) Clark DE, Errington TM, Smith JA, Frierson HF, Jr., Weber MJ, Lannigan DA. The serine/threonine protein kinase, p90 ribosomal S6 kinase, is an important regulator of prostate cancer cell proliferation. Cancer Res 2005; 65(8):3108-16.

(153) Li Q, Zhu GD. Targeting serine/threonine protein kinase B/Akt and cell-cycle checkpoint kinases for treating cancer. Curr Top Med Chem 2002; 2(9):939-71.

(154) Chen JJ, Xia LX, Liu ZG, Liu W, Ji KM. [Cloning, expression and purification of allergen arginine kinase from Periplaneta americana and its allergic

activity]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 2008; 26(5):356-60.

(155) Garcia-Orozco KD, ispuro-Hernandez E, Yepiz-Plascencia G, Calderon-de-la-Barca AM, Sotelo-Mundo RR. Molecular characterization of arginine kinase, an allergen from the shrimp Litopenaeus vannamei. Int Arch Allergy Immunol 2007; 144(1):23-8.

(156) Sookrung N, Chaicumpa W, Tungtrongchitr A, Vichyanond P, Bunnag C, Ramasoota P et al. Periplaneta americana arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. Environ Health Perspect 2006; 114(6):875-80.

(157) Binder M, Mahler V, Hayek B, Sperr WR, Scholler M, Prozell S et al. Molecular and immunological characterization of arginine kinase from the Indianmeal moth, Plodia interpunctella, a novel cross-reactive invertebrate pan-allergen. J Immunol 2001; 167(9):5470-7.

(158) Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clin Exp Allergy 1999; 29(7):896-904.

(159) Astier C, Morisset M, Roitel O, Codreanu F, Jacquenet S, Franck P et al. Predictive value of skin prick tests using recombinant allergens for diagnosis of peanut allergy. J Allergy Clin Immunol 2006; 118(1):250-6.

(160) Matsuo H, Dahlstrom J, Tanaka A, Kohno K, Takahashi H, Furumura M et al. Sensitivity and specificity of recombinant omega-5 gliadin-specific IgE measurement for the diagnosis of wheat-dependent exercise-induced anaphylaxis. Allergy 2008; 63(2):233-6.

(161) Matsuo H, Kohno K, Niihara H, Morita E. Specific IgE determination to epitope peptides of omega-5 gliadin and high molecular weight glutenin subunit is a useful tool for diagnosis of wheat-dependent exercise-induced anaphylaxis. J Immunol 2005; 175(12):8116-22.

(162) Asero R, Jimeno L, Barber D. Component-resolved diagnosis of plant food allergy by SPT. Eur Ann Allergy Clin Immunol 2008; 40(4):115-21.

(163) Pittner G, Vrtala S, Thomas WR, Weghofer M, Kundi M, Horak F et al. Component-resolved diagnosis of house-dust mite allergy with purified natural and

recombinant mite allergens. Clin Exp Allergy 2004; 34(4):597-603.

(164) Ballmer-Weber BK, Scheurer S, Fritsche P, Enrique E, Cistero-Bahima A, Haase T et al. Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. J Allergy Clin Immunol 2002; 110(1):167-73.

(165) Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D et al. Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. J Invest Dermatol 1999; 113(5):830-7.

(166) Constantin C, Quirce S, Poorafshar M, Touraev A, Niggemann B, Mari A et al. Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis. Allergy 2009.

(167) Eigenmann PA. Component-resolved diagnosis in food allergy, are micro-array assays helpful to the clinician? Allergy 2008; 63(11):1519-20.

(168) Valenta R, Twaroch T, Swoboda I. Component-resolved diagnosis to optimize allergen-specific immunotherapy in the Mediterranean area. J Investig Allergol Clin Immunol 2007; 17 Suppl 1:36-40.

(169) Chen Y-H, Lee M-F, Chang S-S, Hwang G-Y. EU678971 Forcipomyia taiwana eukaryotic translation initiation factor mRNA,complete cds. http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=189030778 . 2008.

(170) Lodish H, Berk A, Matsudaira P, Kaiser CA, Krieger M, Scott MP et al. Basic Molecular Genetic Mechanisms. In: Lodish H, Berk A, Matsudaira P, Kaiser CA, Krieger M, Scott MP et al., editors. Molecular Cell Biology. New York: W.H. Freeman and Company, 2004: 101-45.

(171) Valasek L, Nielsen KH, Zhang F, Fekete CA, Hinnebusch AG. Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. Mol Cell Biol 2004; 24(21):9437-55.

(172) Traicoff JL, Chung JY, Braunschweig T, Mazo I, Shu Y, Ramesh A et al. Expression of EIF3-p48/INT6, TID1 and Patched in cancer, a profiling of multiple tumor types and correlation of expression. J Biomed Sci 2007; 14(3):395-405.

(173) Dong Z, Zhang JT. Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. Crit Rev Oncol Hematol 2006; 59(3):169-80.

(174) Zhang L, Pan X, Hershey JW. Individual overexpression of five subunits of human translation initiation factor eIF3 promotes malignant transformation of immortal fibroblast cells. J Biol Chem 2007; 282(8):5790-800.

(175) Marchetti A, Buttitta F, Pellegrini S, Bertacca G, Callahan R. Reduced expression of INT-6/eIF3-p48 in human tumors. Int J Oncol 2001; 18(1):175-9.

(176) Asano K, Merrick WC, Hershey JW. The translation initiation factor eIF3-p48 subunit is encoded by int-6, a site of frequent integration by the mouse mammary tumor virus genome. J Biol Chem 1997; 272(38):23477-80.

(177) Oh CK, Filler SG, Cho SH. Eukaryotic translation initiation factor-6 enhances histamine and IL-2 production in mast cells. J Immunol 2001; 166(5):3606-11.

表 與 圖

表一 台灣鋏蠓叮咬過敏之盛行率

反　　應	人　數 (n=372)	百分比
無過敏反應	152	40.9%
有過敏反應	220	59.1%
過敏反應類型		
純立即型 Solely immediate reaction	30	14%
純延遲型 Solely delayed reactions	95	43%
合併型 Immediate reaction+delayed reactions	95	43%

表二 台灣鋏蠓過敏反應之臨床表徵與皮膚試驗結果

Table 2. Clinical characteristics of hypersensitive reactions to *F. taiwana*

Subject	Age	Sex	Symptoms	Skin test/kU/L	Atopy	Autoimmune disease
1	42	F	I, D	4+/16.39	AR	–
2	23	F	I, D	1+/9.28	–	SLE
3	28	M	I, D	4+/22.89	–	–
4	37	M	I	3+/11.94	–	–
5	14	M	I, D	2+/16.43	AR	–
6	24	F	I, D	1+/8.25	–	–
7	53	M	I, D	4+/10.56	–	SLE
8	39	F	I, D	3+/19.34	–	–
9	18	M	I	2+/21.9	AR, AS, AD	–
10	37	F	I, D	1+/22.56	–	–
11	29	F	I, D	2+/29.98	–	SLE
12	60	F	I	2+/11.5	–	–
13	45	F	D	–/0.65	–	–
14	24	F	D	–/8.25	–	–
15	36	F	D	–/1.40	AR	–
16	50	F	D	–/3.04	AR	–
17	29	M	D	–/6.78	–	–
18	45	F	D	–/0.72	–	–
19	22	F	D	–/5.32	AR	–
20	37	F	D	–/4.94	–	–
21	24	F	D	–/1.12	AR	–

I, immediate reaction; D, delayed reaction; AS, asthma; AR, allergic rhinoconjunctivitis; AD, atopic dermatitis; SLE, systemic lupus erythematous.

表三 受試者基本資料

Reactions after midge bite	Immediate allergic reaction ±delayed reaction	Solely delayed reaction	Non-allergic control
Number	20	12	30
Age (mean±SD)	32.1±2.47	37.75±2.54	29.1±2.01
Gender	4M,16F	2M,10F	15M,15F
SPT to crude midge extract (mean wheal size±SD /mean erythema size±SD)	6.24±0.38 / 15.82±2.12mm	0.25±0.18 / 0.33±0.22mm	0±0 / 0.09±0.06mm

M= male; F=female；SD=standard deviation

Table 4. Other cytokine secretion after stimulated with crude midge extract (3ug/ml)

	IL-8 (pg/ml)		IL-2 (pg/ml)		IL-1beta (pg/ml)		IL-4 (pg/ml)		IL-5 (pg/ml)		IL-12p70 (pg/ml)		TNF-beta(pg/ml)	
	A	NA	A	NA	A	NA	A	NA	A	NA	A	NA	A	NA
48h	13102.38	11200.43	60.27	70.52	80.81	39.26	UD	UD	UD	UD	UD	UD	UD	UD
96h	9952.23	11729.97	58.78	63.97	100.19	27.72	UD	UD	UD	UD	UD	UD	UD	UD
168h	10257.73	11838.76	73.41	84.84	62.05	24.5	UD	UD	UD	UD	UD	UD	UD	UD

A:midge-allergic; NA:midge nonallergic; UD: undetectable

表五 以電噴灑游離子原串聯式質譜儀ESI-MS/MS 鑑定台灣鋏蠓主要過敏原

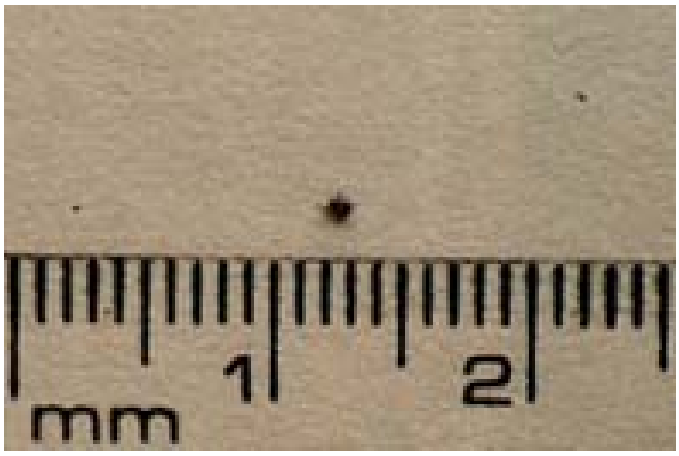
Allergen*	pI*	Matching identity	Species	GenBank access. No.	MW/pI (GenBank)	Matching residue sequence
For t 1 (24 kDa)	5.8	Triosephosphate isomerase (EC 5.3.1.1)	*A. merus*	gi:1857455	21.86/5.33	^{104}VIACIGETLQER^{115} ^{156}TATPEQAQEVHAALRK^{171} ^{187}IQYGGSVTAANCR^{199}
			T. molitor	gi:22090453	26.66/6.0	^{84}DVGADWVILGHSERR^{97} ^{122}VIACIGETLEER^{133} ^{148}AIAAKVNDWSNVVIAYEPVWAIGTGK^{173}
			O. togoi	gi:1857457	21.91/5.30	^{104}VIACIGETLQER^{115} ^{66}DVGANWVIIGHSER^{79}
			C. vittatus	gi:1857453	16.42/5.39	^{171}KWFTDNVSADVSASIRIQYGGSVTAANCR^{199} ^{104}VIACIGETLQER^{115}
For t 2 (35 and 36 kDa)	8.6	Fructose	*H. coagulata*	gi:46561746	39.68/7.56	^{88}ADDGTPFVELLKK^{100} ^{157}NTPSYQAILENANVLAR^{173}
	8.7	1,6-bisphosphate aldolase (EC 4.1.2.13)	*A. yamamai*	gi:45330818	39.67/7.59	^{157}NTPSYQAILENANVLAR^{173} ^{183}IVPIVEPEVLPDGEHDLDR^{201}
For t 3 (64 kDa)	5.2	Slow muscle myosin S1 heavy chain	*H. americanus*	gi:37925239	58.53/5.22	^{16}QRIATELEDMQIEVER^{31} ^{352}AKKAMVDAAR^{361} ^{478}QIEEAEEIAALNLAK^{492}

*Experimental molecular weight and pI.

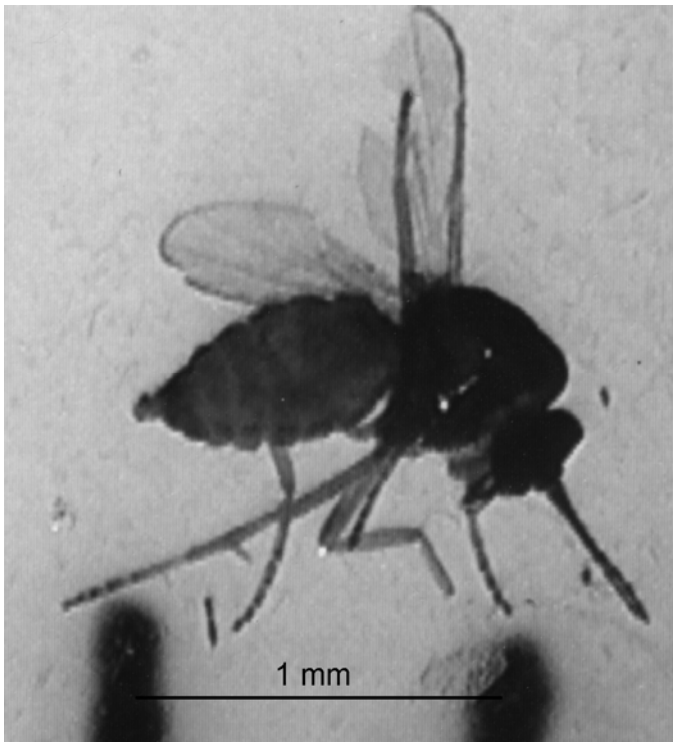
圖一 我們使用東海大學生命科學系陳錦生老師實驗室設計之捕蠓器，以人體誘集法活捉台灣鈸蠓雌蟲作為實驗材料

圖二

A.台灣銨蠓實體大小

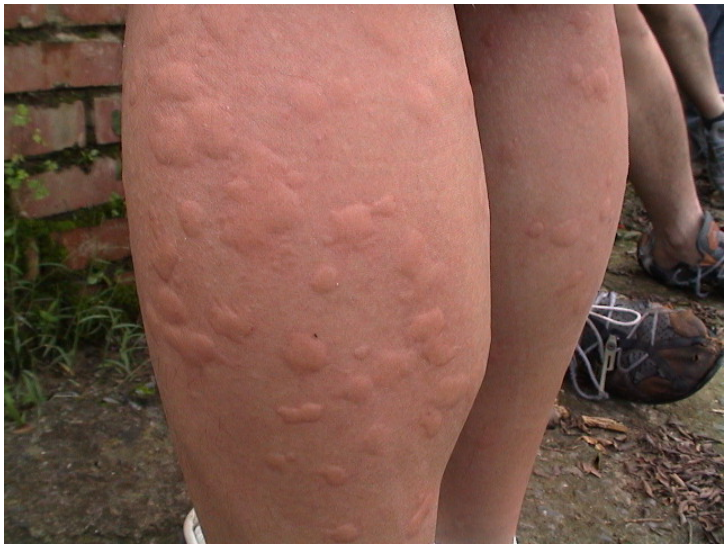


B.10 倍顯微鏡下之台灣銨蠓

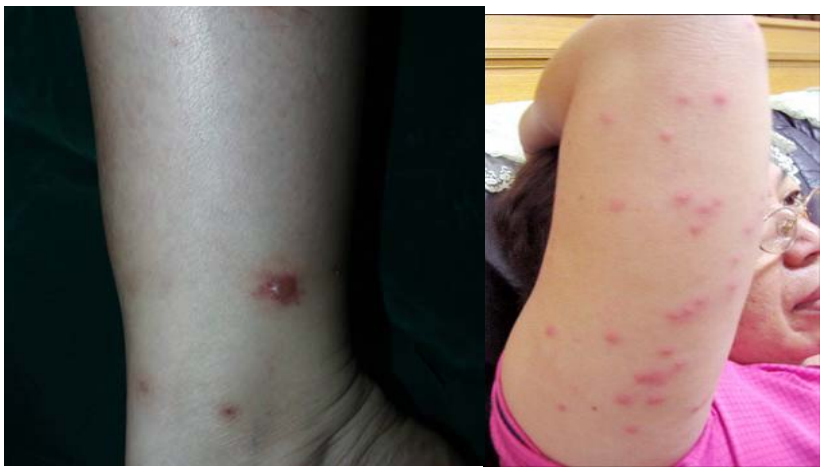


圖三 台灣鋏蠓叮咬之過敏反應

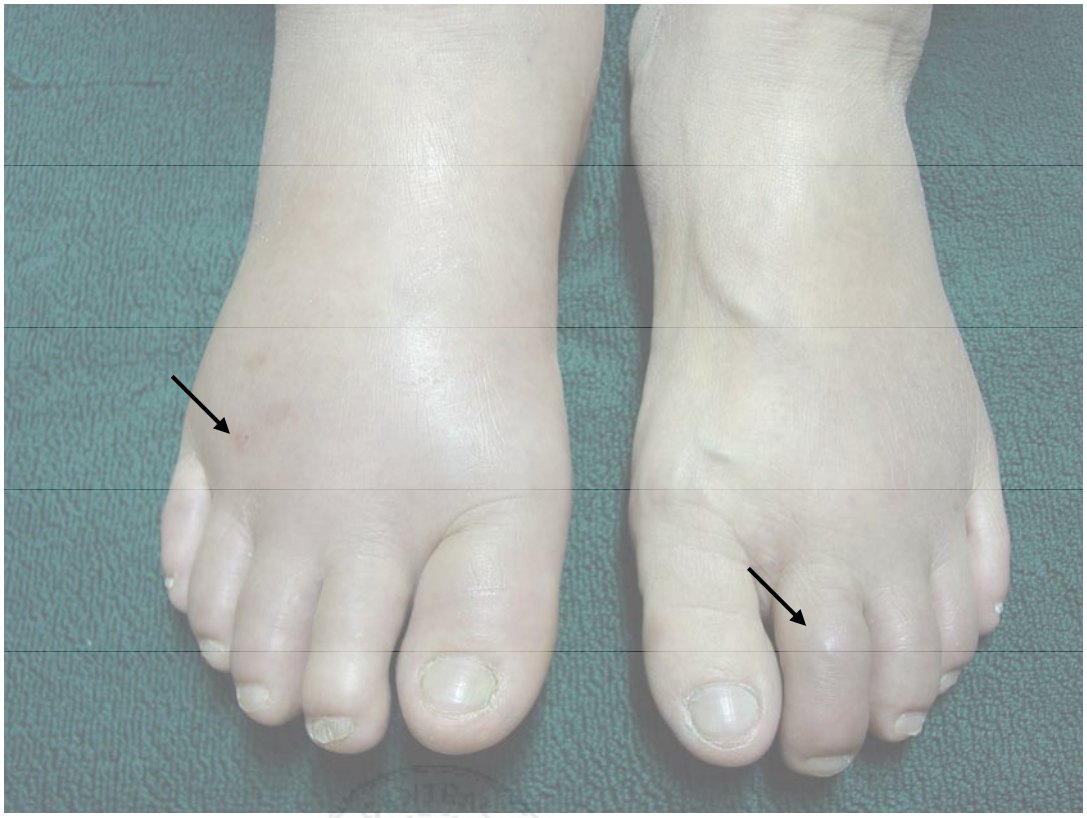
A. 立即型皮膚反應：患者於被叮咬一小時內，產生局部明顯的紅腫膨疹，其大小可由直徑 2 公分至大於 10 公分以上



B. 純延遲型皮膚反應 : 患者於被叮咬後 6-24 小時產生劇癢之丘疹或水泡，有些人之患處中央還會壞死，持續數週至數月方消。

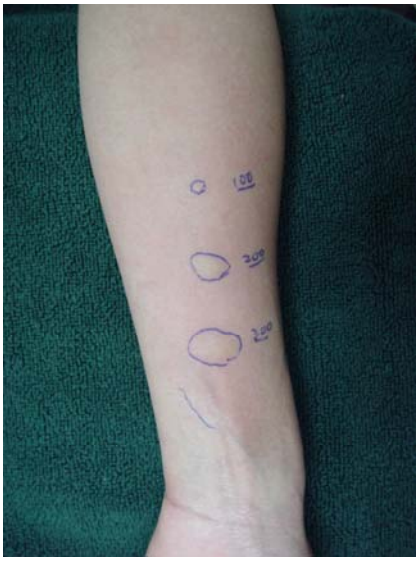


C. 立即型加延遲型皮膚反應：患者於被叮咬一小時內，產生局部明顯的紅腫，紅腫的程度常被誤診為蜂窩組織炎。除紅腫外，叮咬處還會合併水泡或丘疹， 箭頭標示處有水泡形成。(本張照片由謝祖怡醫師提供)

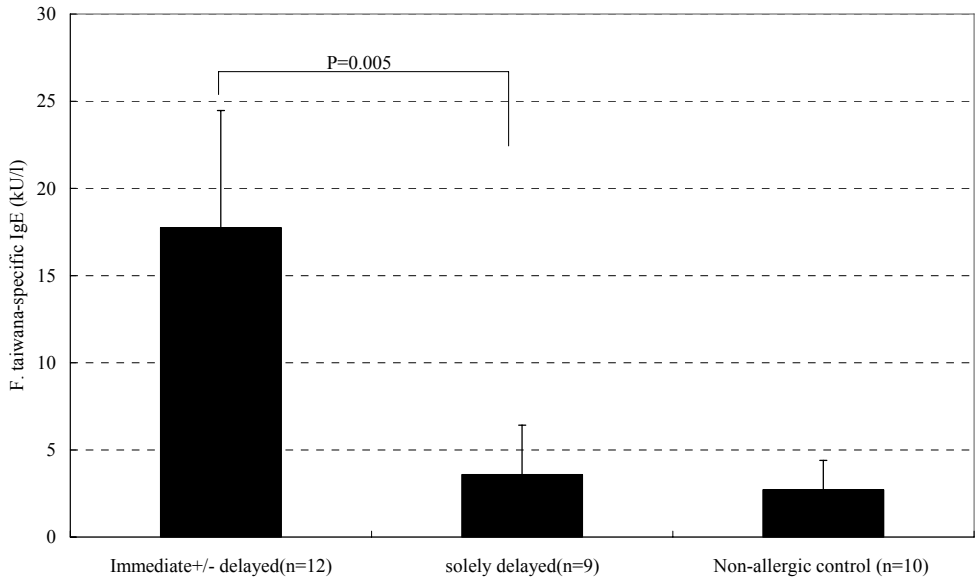


圖四 **(A)**台灣鈸蠓萃取液可引立即型病患陽性之皮膚試驗。以台灣鈸蠓粗粹物 100μg/m、200μg/ml、及 300μg/ml 做過敏原皮膚試驗，立即型台灣鈸蠓過敏者會出現典型之膨疹紅暈(wheal-and-flare)反應，且有 dose-dependent 之現象。(B)以 ELISA 測定受試者血清中之台灣鈸蠓專一型免疫球蛋白 E。立即型患者為 17.75±6.72 kU/l，純延遲型患者為 3.58±2.84 kU/l；無過敏反應者則為 2.71±1.69 kU/l。

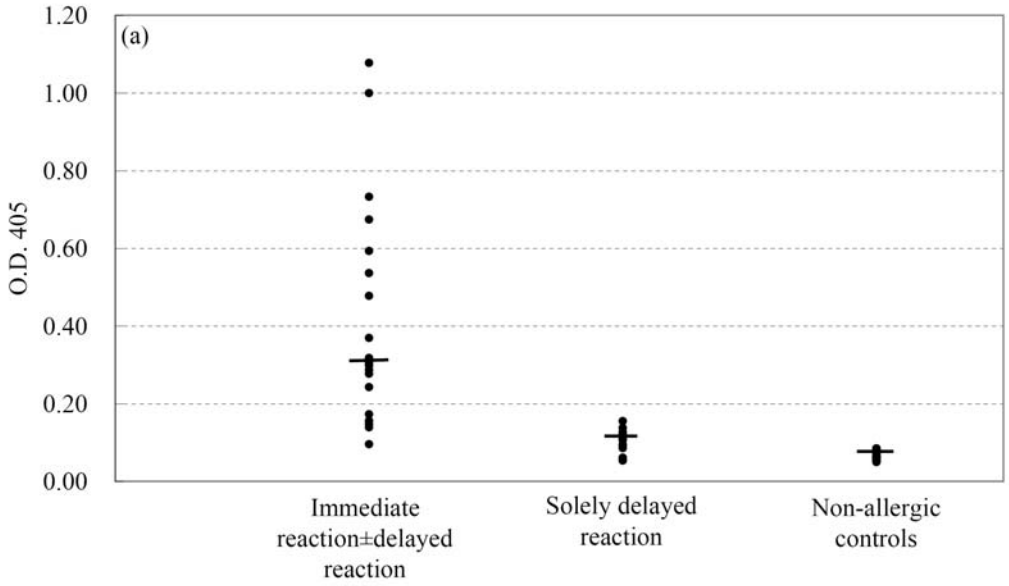
A.

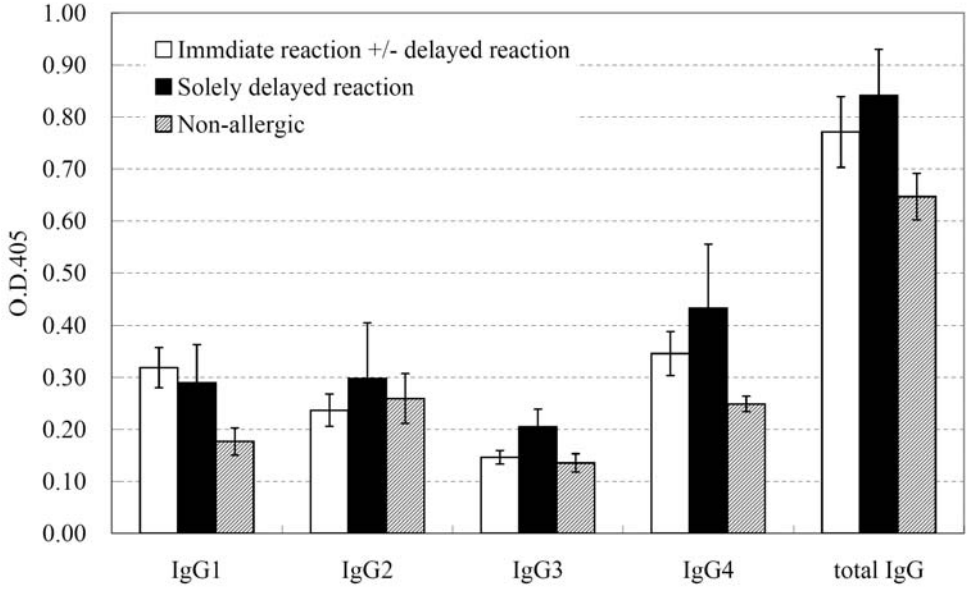


B

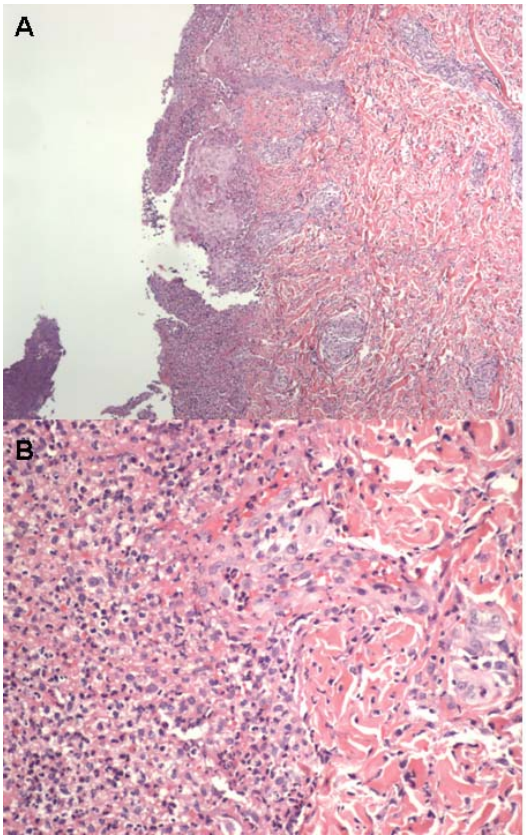


圖五 以酵素聯結免疫吸附法測定三組不同叮咬反應受試者之(a)台灣鋏蠓專一型免疫球蛋白 E 與(b)台灣鋏蠓專一型免疫球蛋白 G 及其亞型。實驗結果為 20 名立即型過敏反應者(immediate reaction±delayed reaction)、12 名純延遲型過敏反應者(solely delayed reaction)與 30 名對照組(non-allergic controls)血清所測數值之平均值±標準誤。

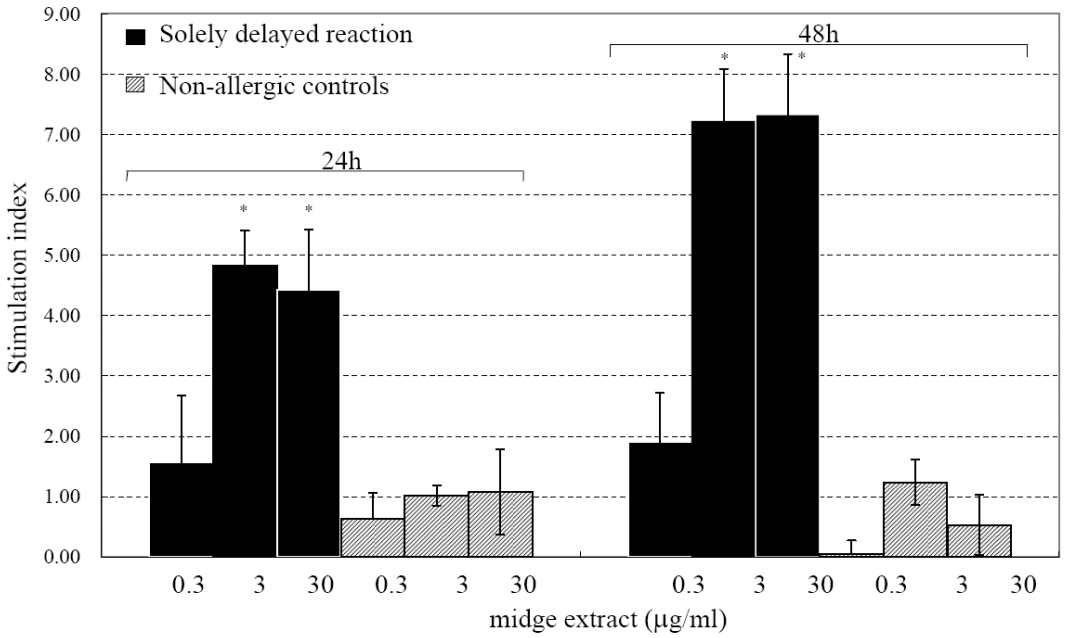




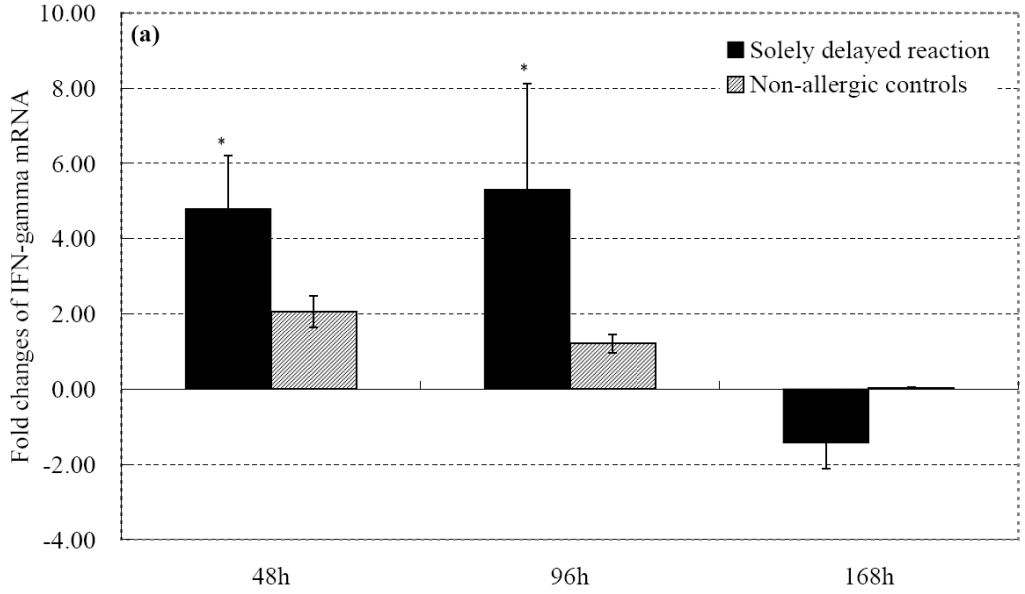
圖六 延遲型台灣鋏蠓叮咬過敏反應皮膚病灶之組織學所見。受試者皮膚檢體在台灣鋏蠓叮咬 24 至 48 小時後切片，以蘇木精(hematoxylin)與伊紅(eosin)染色並於(A) 40 倍 (B) 400 倍光學顯微鏡下觀察。

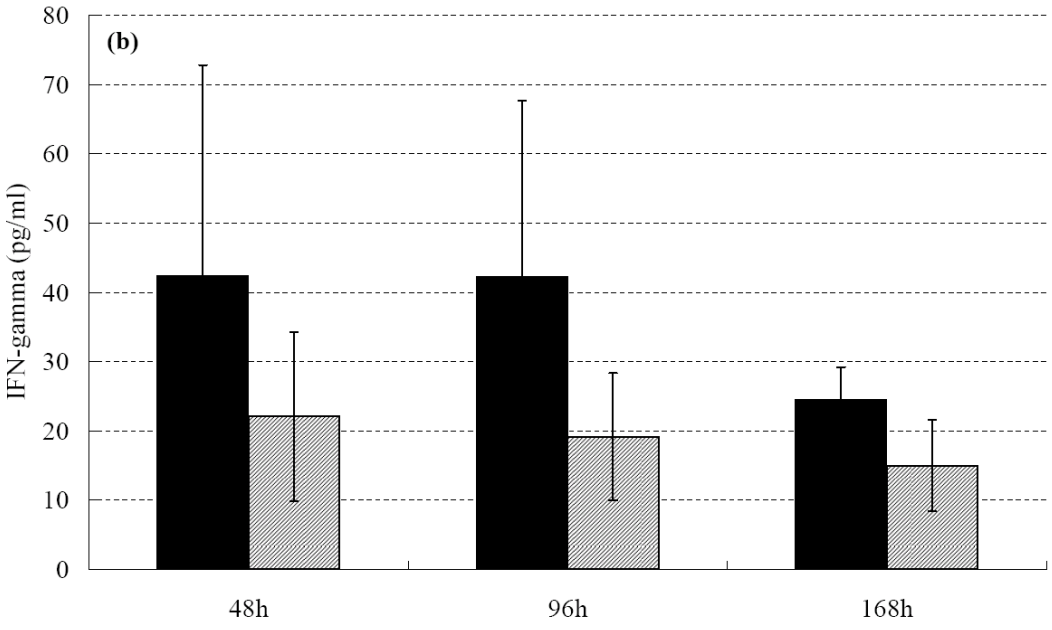


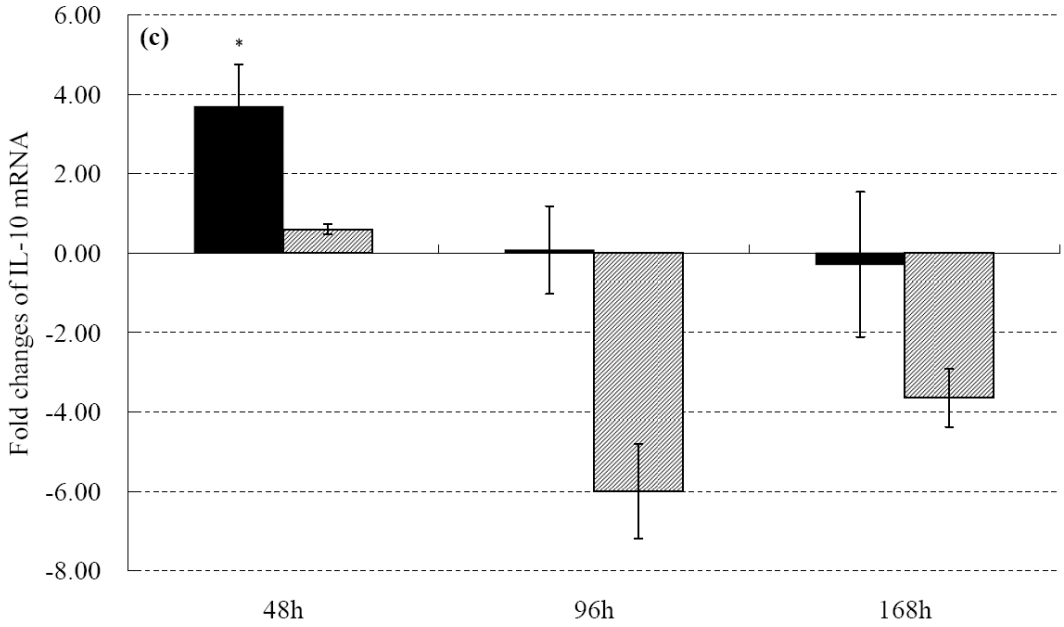
圖七 六名純延遲型過敏反應(solely delayed reaction，以黑色實心柱表示)與六名無台灣銳蠓過敏對照組(non-allergic controls，，以斜線半空心柱表示)受試者之周邊單核球以不同濃度之台灣銳蠓粹取物(midge extract)刺激後之增生反應。Stimulation index計算方法為：與台灣銳蠓萃取物共培養細胞之O.D. 570nm值 ÷ 同一時間點僅有培養基培養細胞之O.D. 570nm值。實驗數據以六次實驗之平均值±標準誤表示。* 表示與無過敏之對照組相比其 P值<0.05。

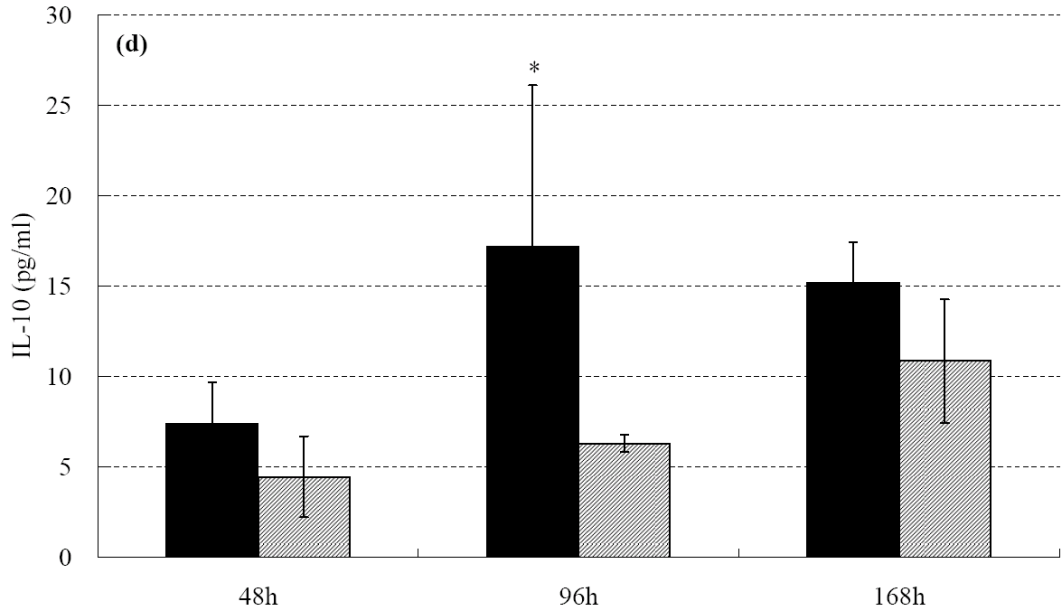


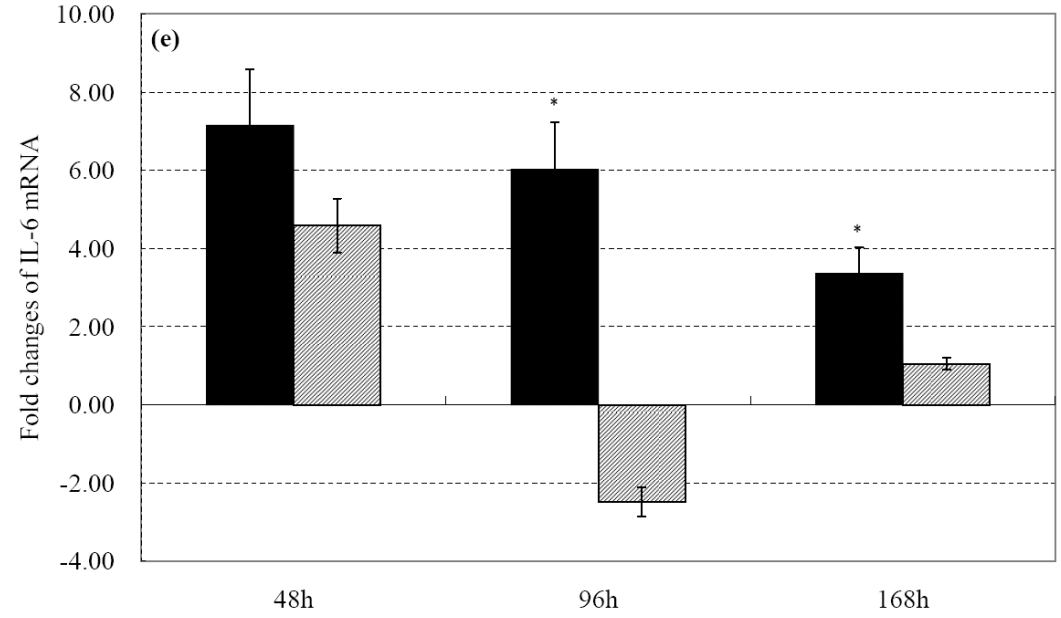
圖八　六名台灣鋏蠓純延遲型過敏反應(solely delayed reaction)與六名對照組(non-allergic controls)受試者周邊單核球細胞激素訊息核糖核酸(rnRNA)與蛋白質之表現。受試者之周邊單核球與3μg/ml之台灣鋏蠓粹取物共培養不同時間後，細胞激素之訊息核糖核酸以及時聚合酵素連鎖反應(real-time PCR)而蛋白質以流式細胞微珠免疫法測定之。(a)丙型干擾素(IFN-gamma)訊息核糖核酸(b)丙型干擾素蛋白質(c)第十介白質(IL-10)訊息核糖核酸(d)第十介白質蛋白質(e)第六介白質(IL-6)訊息核糖核酸(f)第六介白質蛋白質(g)甲型腫瘤壞死因子(TNF-alpha)訊息核糖核酸(h)甲型腫瘤壞死因子蛋白質。實驗數據以六次實驗之平均值±標準誤表示。*表示與無過敏之對照組相比其 P 值<0.05。

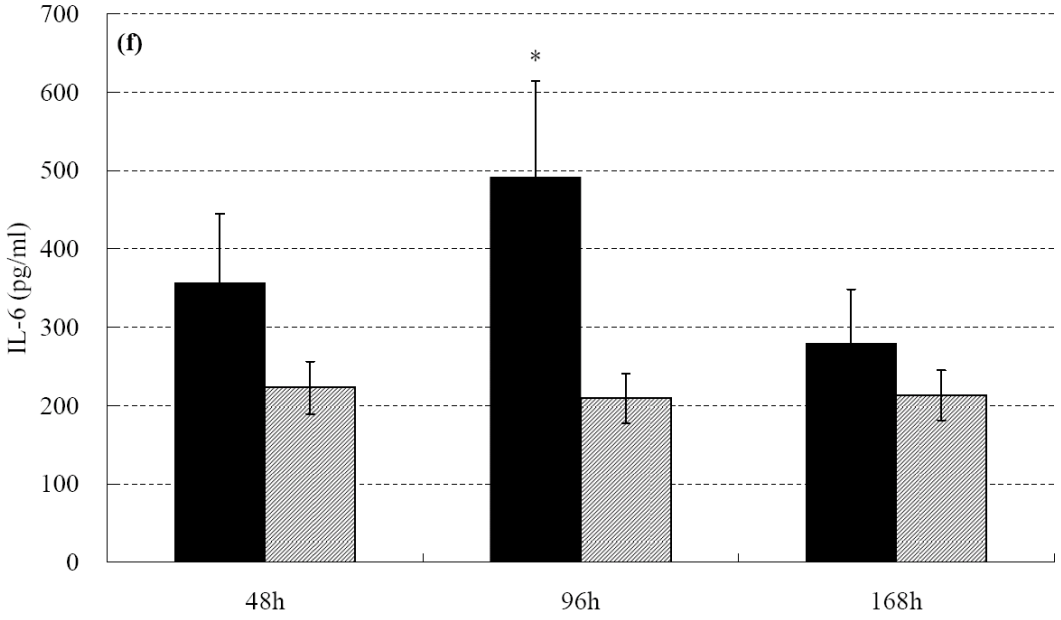

(a)
Solely delayed reaction
Non-allergic controls
Fold changes of IFN-gamma mRNA
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
*
*
48h
96h
168h

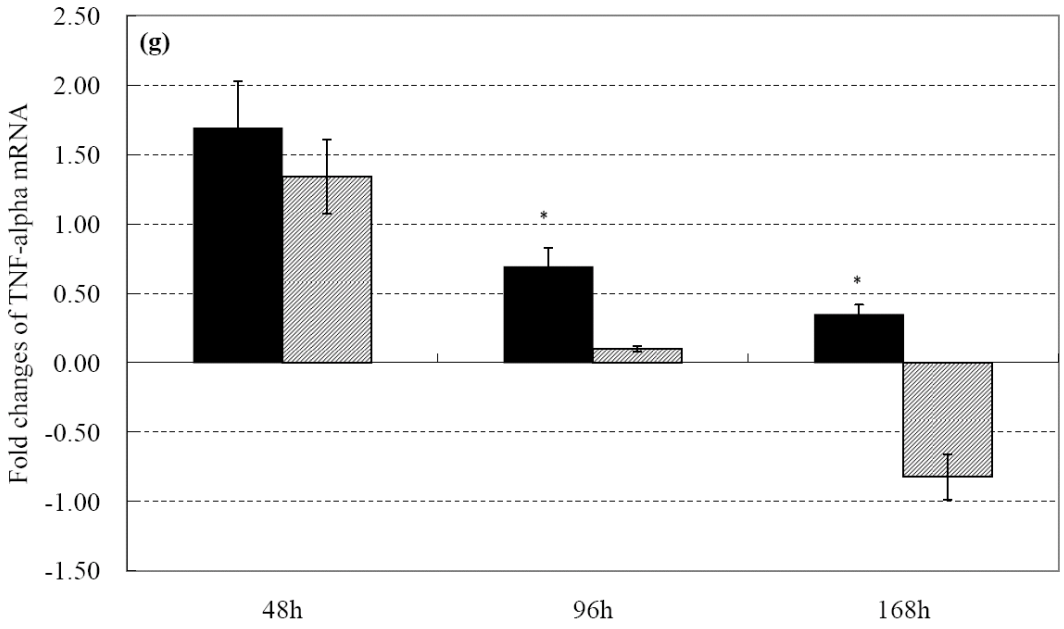

(b)
IFN-gamma (pg/ml)
80
70
60
50
40
30
20
10
0
48h
96h
168h

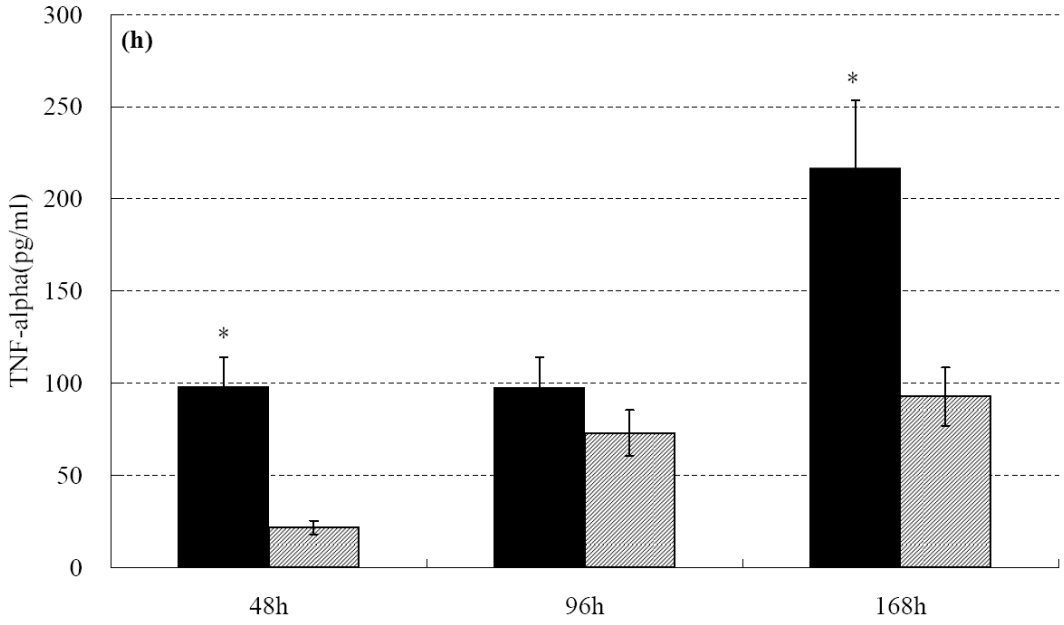

(c)
*
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
Fold changes of IL-10 mRNA
48h
96h
168h


(d)
*
30
25
20
15
10
5
0
IL-10 (pg/ml)
48h
96h
168h

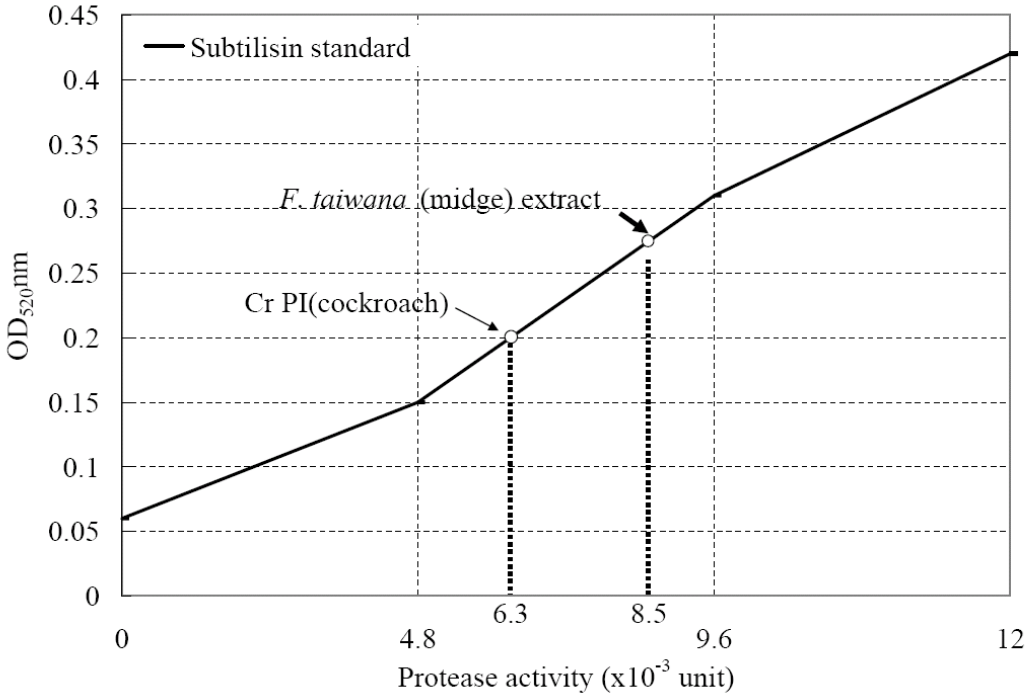

(e)
Fold changes of IL-6 mRNA
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
*
*
48h
96h
168h


(f)
IL-6 (pg/ml)
700
600
500
400
300
200
100
0
*
48h
96h
168h

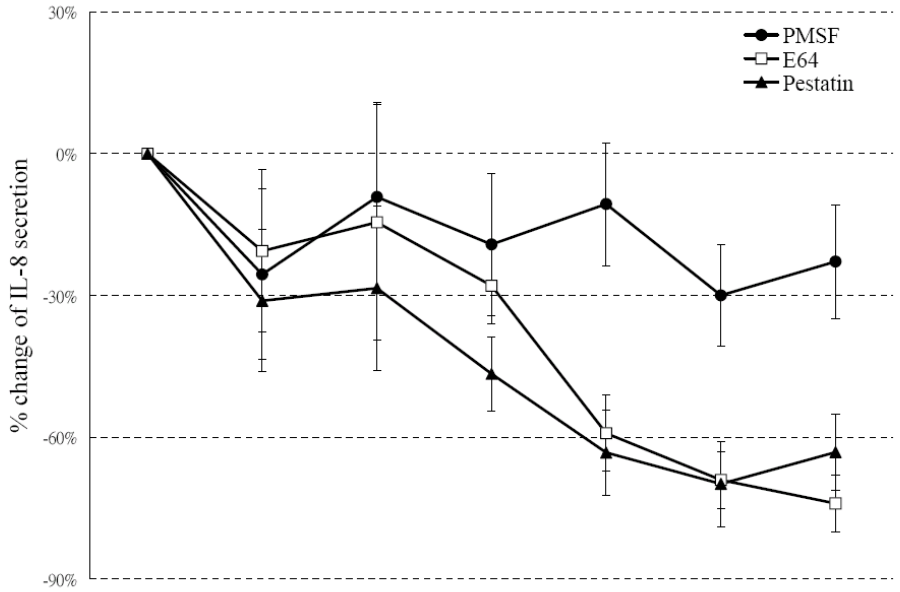

(g)
Fold changes of TNF-alpha mRNA
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
*
*
48h
96h
168h


(h)
TNF-alpha(pg/ml)
300
250
200
150
100
50
0
*
*
48h
96h
168h

圖九 以 Azocoll 法測定台灣鈸蠓粹取物(20μg/ml)之蛋白腖活性。粗箭號所指為台灣鈸蠓粹取物，其蛋白腖活性約當於 8.5 x10^{-3} units；細箭號所指為蟑螂過敏原 Cr P I，其蛋白腖活性約當於 6.3 x10^{-3} units。

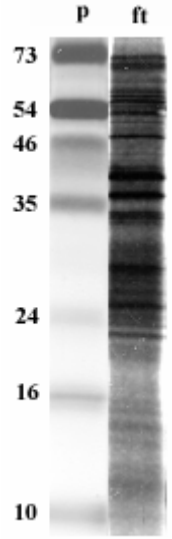


圖十 蛋白腖抑制劑(protease inhibitors)在 A549 細胞上對台灣銨蠓粹取物刺激分泌之第八介白質之影響。台灣銨蠓粹取物(100μg/ml)先各與不同種類不同濃度之蛋白腖抑制劑，PMSF (絲胺酸 serine 與半胱胺酸 cysteine 蛋白腖抑制劑)、E64(木瓜蛋白 papain 與半胱胺酸 cysteine 蛋白腖抑制劑)或 pepstatin(天門冬胺酸 aspartic 蛋白腖抑制劑)，共培養 15 分鐘後再與 A549 細胞共培養 24 小時。三種蛋白腖抑制劑之濃度如圖九下方之表格所示。實驗數據以三次相同實驗之平均值±標準誤表示。



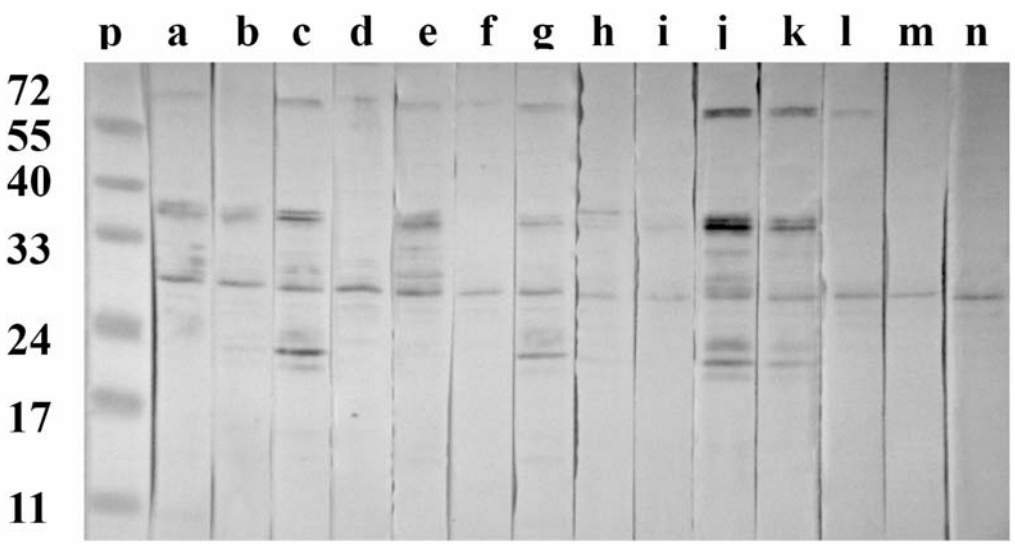
Midge extract (μg/ml)	100	100	100	100	100	100	100
with PMSF (nM)	0	0.01	0.05	0.1	0.2	0.3	0.4
or E64 (ng)	0	5	10	20	50	100	200
or Pepstatin (ng)	0	5	10	20	50	100	200

圖十一　台灣鈸蠓粹取物蛋白質分析。台灣鈸蠓粗萃取物經蛋白質電泳 SDS-PAGE 分析，至少有 33 種不同之蛋白質組成，其分子量由 10 到 70 kDa。P 欄為蛋白質標記(protein markers)，其單為為 kDa，ft 欄為台灣鈸蠓粹取物。



圖十二　免疫轉漬法發現台灣鋏蠓粹取物中有 11 個蛋白質可與台灣鋏蠓立即型過敏反應病患血清中的 IgE 結合。(a) p 欄為蛋白質標記(protein markers)，其單為為 kDa。a 至 l 欄為台灣鋏蠓過敏病患，m 欄為無台灣鋏蠓過敏之對照受試者，n 欄為完全無過敏之對照受試者。29kDa 處有一 non-specific binding band。 (b) 22-、24-、 35-、36-及 64-kDa 之蛋白質過敏原可分別與 50%、50%、75%、66.7% 及 75%之病患血清反應，為台灣鋏蠓主要過敏原。

(a)

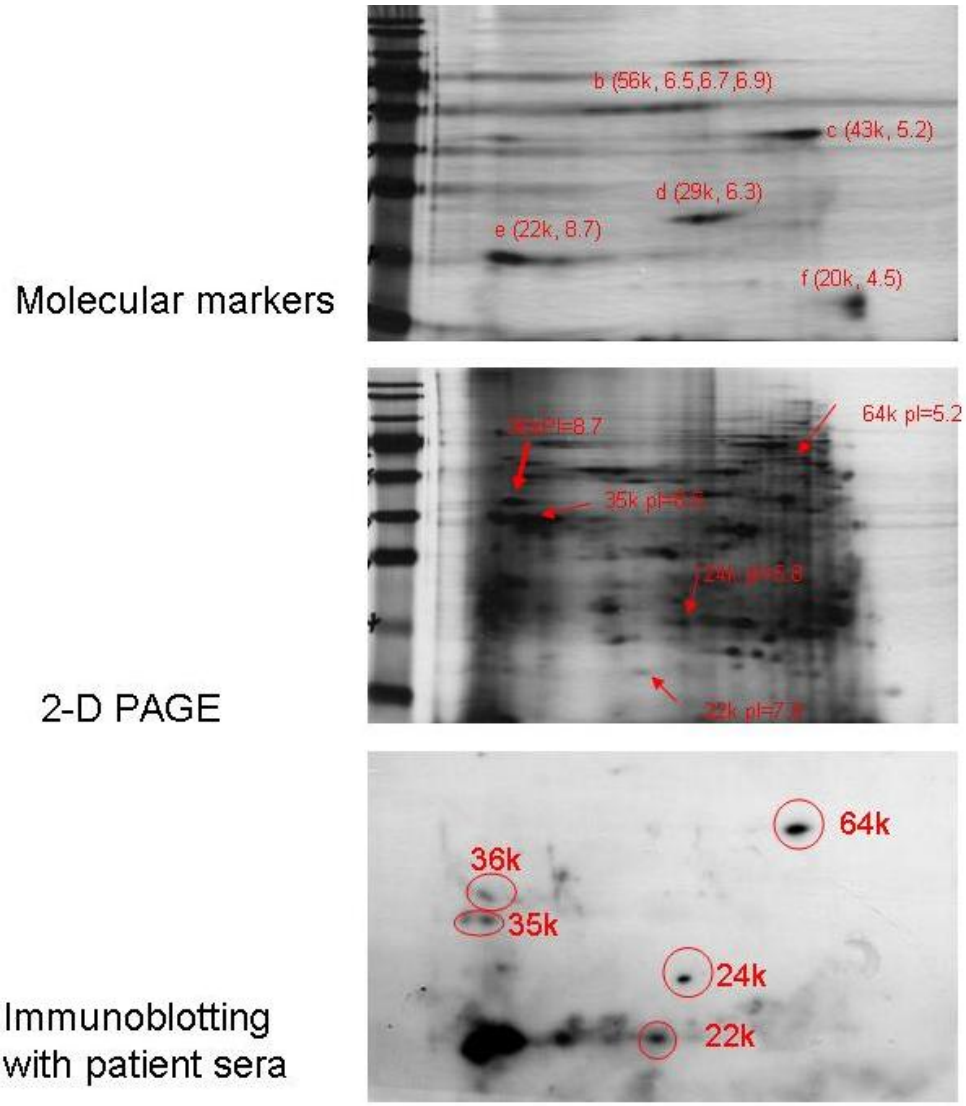


(b)

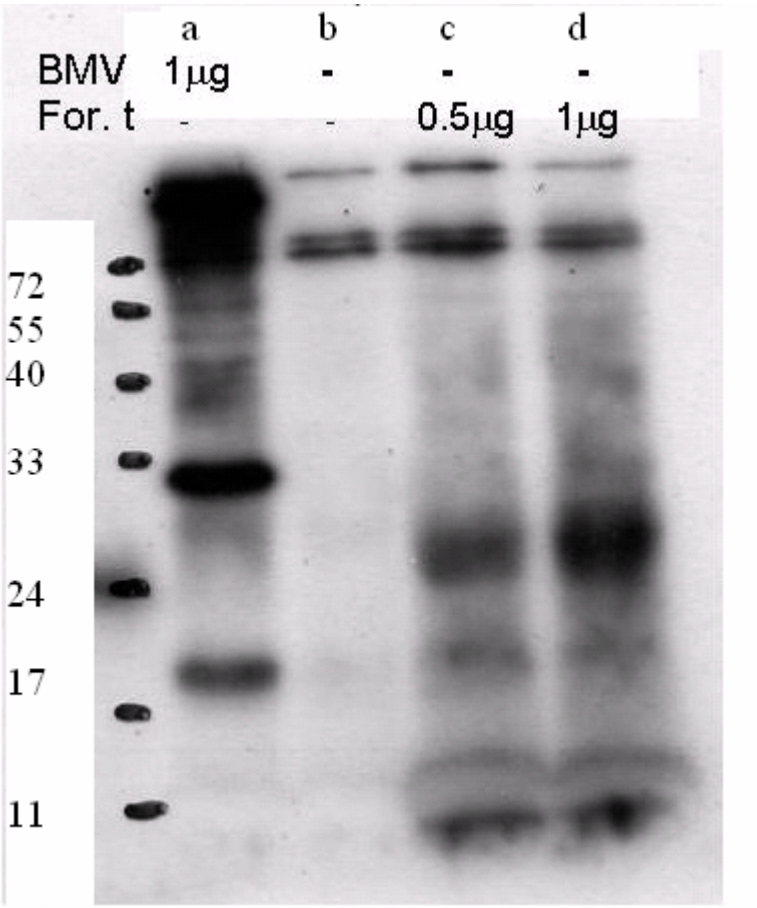
	Molecular weight (kD)										
subject	8	20	22	24	29	30	31	35	36	37	64
1	X										
2				X	X		X	X	X		X
3			X					X	X		
4		X	X	X	X		X	X	X		X
5				X		X					X
6					X	X	X	X	X		X
7											X
8		X	X	X				X	X		X
9			X					X	X	X	
10								X			
11		X	X	X	X		X	X	X		X
12		X	X	X				X	X		X
n											X
w											
Y	8%	33%	50%	50%	33%	17%	33%	75%	67%	8%	75%

Y=frequency recognized by specific IgE

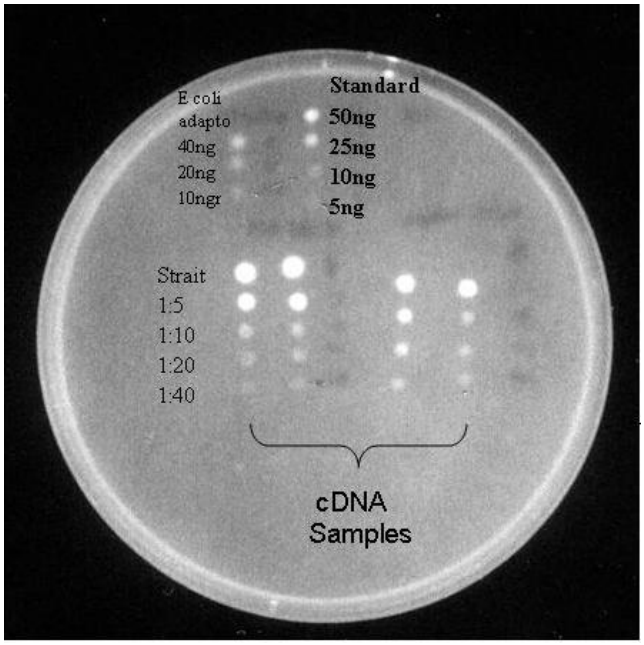
圖十三 蛋白質二維電泳分析台灣鈇蠓過敏原。二維電泳顯示 22-、24-、35-、36-及 64 kDa 蛋白質之等電位點(Isoelectric point, pI) 分別為 8.7、5.8、8.6、8.7 及 5.2。



圖十四　台灣銳蠓訊息核糖核酸(mRNA)經體外轉譯作用之蛋白質產物以 SDS-PAGE 膠電泳分析其結果。a 欄為陽性對照組 BMV mRNA；b 欄為陰性對照組；c 欄為 0.5μg 台灣銳蠓 mRNA 體外轉譯所得之蛋白質；d 欄為 1μg 台灣銳蠓 mRNA 體外轉譯所得之蛋白質

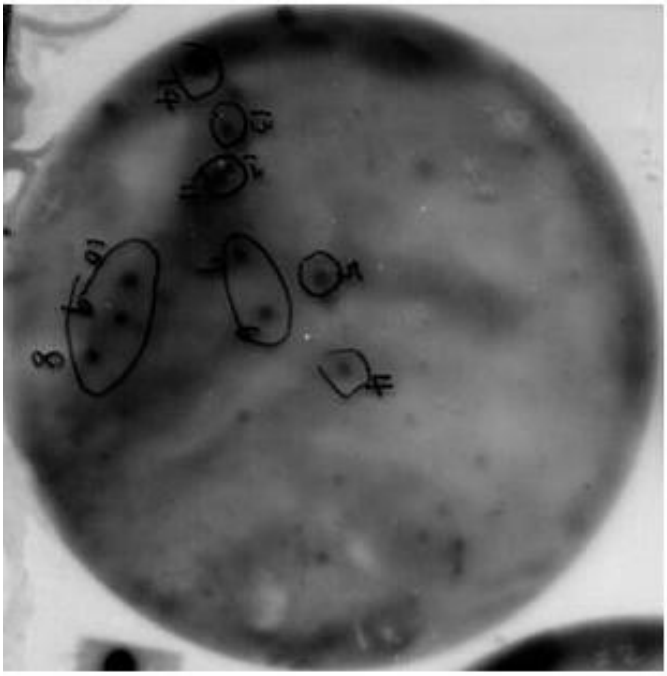


圖十五 構築台灣鋏蠓 cDNA 基因庫前以 ethidium bromide plate assay 半定量 cDNA 產物，約 200ng/μl。

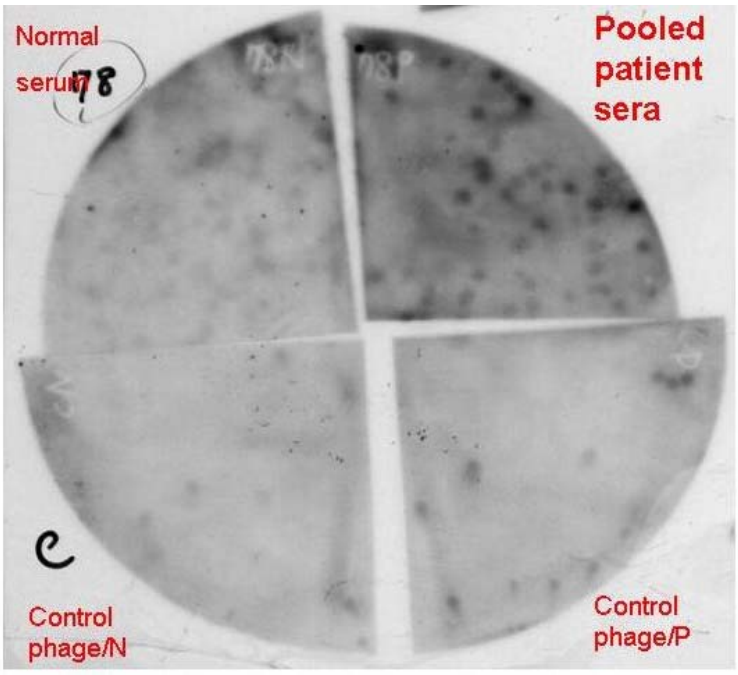


圖十六 以病患血清做 cDNA 基因庫之選殖及次選殖(a)初步篩選與病患血清反應有陽性冷光訊號的選株 (b)經過次選殖後做二次確認。本圖為一代表性之 IgE 結合選株(IgE-binding clone)。

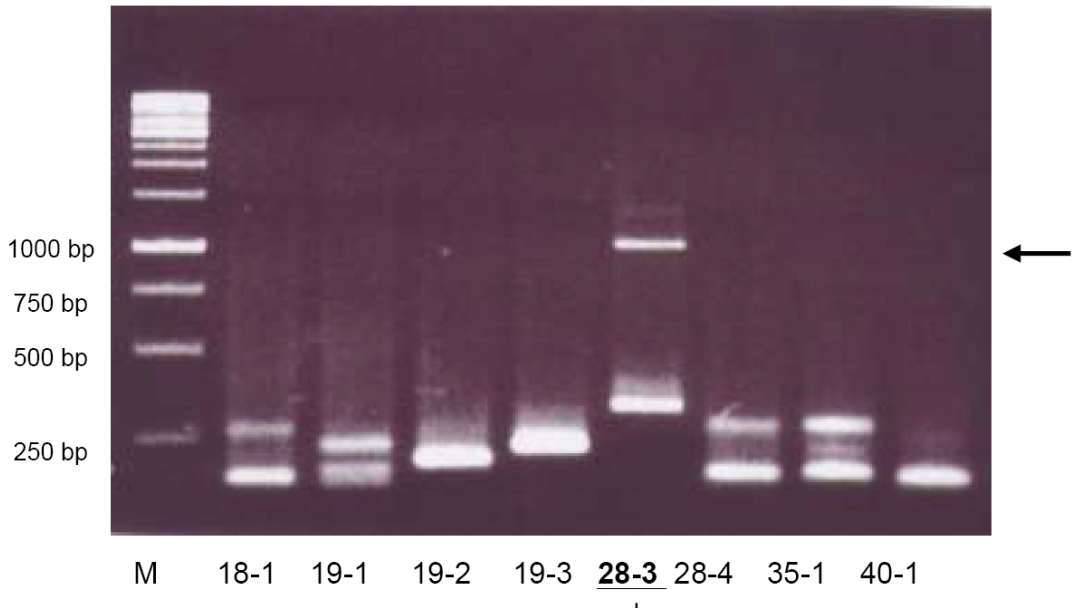
(a)



(b)



圖十七 以 T3 及 T7 引子將 IgE binding clone 做 PCR 增幅



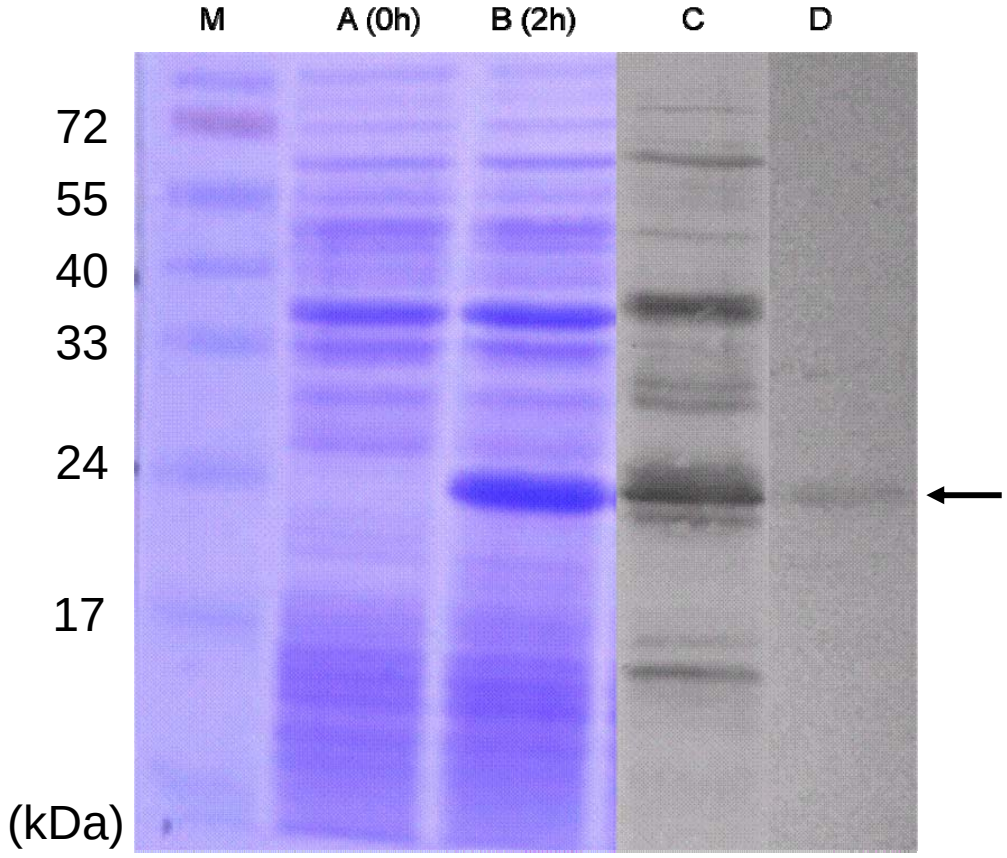
圖十八 編號 TF28-3 之選株經核苷酸定序，共有 898 個核苷酸，為一 partial clone，其 open reading frame 有 357 個核苷酸，經轉譯後有 118 個胺基酸。其換算預估之蛋白質之分子量為 13.67kDa，等電點為 4.84。經向 WHO/IUIS 登錄，選株 TF28-3 正式命名為 For t 1.0101 。

```
  1 GGCACGAGGGTGGATGAGATAAAGGGACATCCCTTCTTCCGCGAAATAGAC
  1 G  T  R  V  D  E  I  K  G  H  P  F  F  R  E  I  D

 52 TGGACGAAGGAGTTGCGGAACCAGAAGGCGCCATACGAACCAAAAATAAAA
 18 W  T  K  E  L  R  N  Q  K  A  P  Y  E  P  K  I  K

103 TACCCAACAGACACCTCGAACTTTGACCCCATAGACCCCGACAAGCTGCAC
 35 Y  P  T  D  T  S  N  F  D  P  I  D  P  D  K  L  H

154 GACTCCTCGAACGCCGACGACTACATGATCGAGGAGTTCTTTGGGAGCGGC
 52 D  S  S  N  A  D  D  Y  M  I  E  E  F  F  G  S  G

205 AAACCGTGCCATCACGGCTTCTTCGAATTTACATTTCGTCGCTTCTTCGAC
 69 K  P  C  H  H  G  F  F  E  F  T  F  R  R  F  F  D

256 GACGATGCGGACTGTAAAATATCCCTCAACCATGGAAATAGTGGCGATAGA
 86 D  D  A  D  C  K  I  S  L  N  H  G  N  S  G  D  R

307 AACGACAATAACAATAGCAATGACAATCAATCTGGAGCAATTTATGTGTAG
103 N  D  N  N  N  S  N  D  N  Q  S  G  A  I  Y  V  *
    TGAAACTTAGGGAGACGAAGGGAGAGTTTTATCGTTTTAAATCAAGTAGAG
    CAAGCTAGCCTGTAAATTAGCGTAGGTTTTCGTTTGTTTTCTTTTTTTTTA
    TAATTTTAATTTTCATCATTGTATACATATATTTCGACTAGTCAATCTCGT
    ATGTTCATTATTTTATTGTATGTATAATTTTACTGATAAAGAAACAAACAG
    AGAGTGCAGGAACAAGCTTGGAGACGAAACAAAAATTCGCATGAATATGTA
    ATTATAAAATATTTAATATATGTATCATCAAGTACAGCAAAGTAAGTGAAA
    ATGGGGTTGCTACTTTGGCCATCAATGCTCCAATCCAACCCTTCGTAGATA
    AAAAATCTAGAGTATTAGAAATATTTAATAAAATTTATGTATTTTAACGGT
    TAAATTAACATTATTGCTATTTGCTATGTTTGCGCCTTTTAAGAAGAAACC
    CTATTATTATACAATGTACTGTATAACAATAATTAAGAACGTAATAAAATT
    TGAAAATGAAAAAAAAAAAAAAAAAAAAAA
```

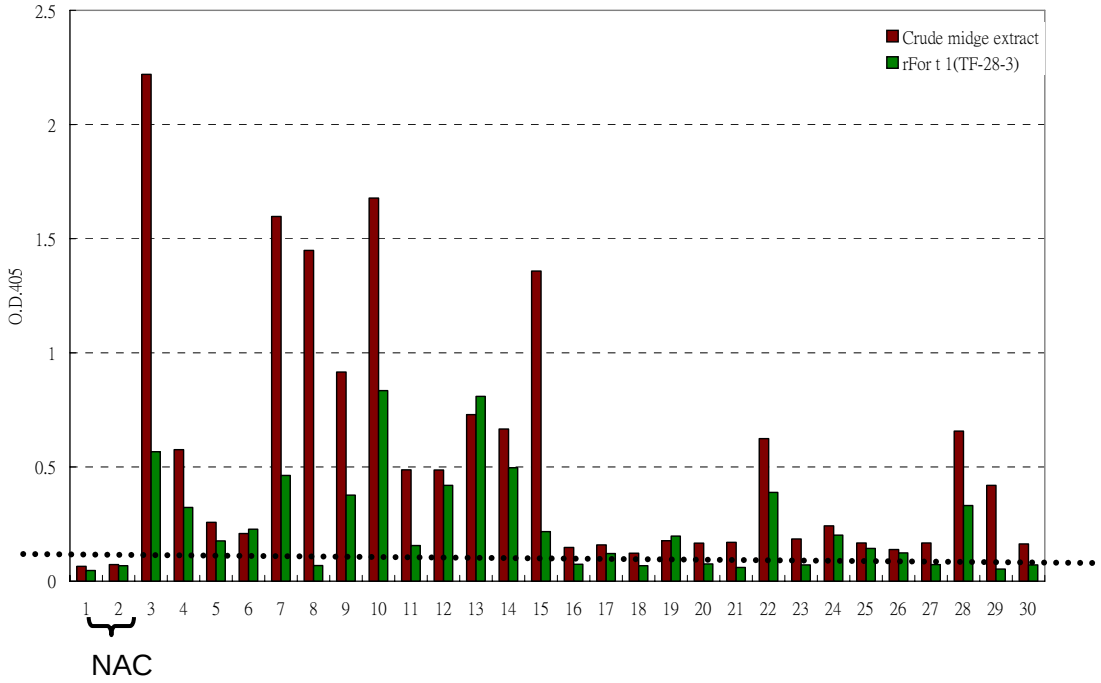
圖十九　重組過敏原 For t 1.0101 經 NCBI blastp 比對結果，與埃及斑蚊之 serine/threonine protein kinase 有 65.3%之序列相似性。

```
             10        20        30        40        50        60
For     GTRVDEIKGHPFFREIDWTKELRNQKAPYEPKIKYPTDTSNFDPIDPDKLHDSSNADDYM
        :  :..::.::::: ::.::.:::: ::::::::: :::::::::: ::.:::  .. .
Aed     GRDVNKIKSHPFFRTIDFTKDLRNQPAPYEPKIKYATDTSNFDPIDPGKLQDSSCEESGQ
             10        20        30        40        50        60

              70
For     -IEEFFGSGKPCHHG
         ... : :.:: :::
Aed     SFDDVFDSSKPFHHG
             70
```

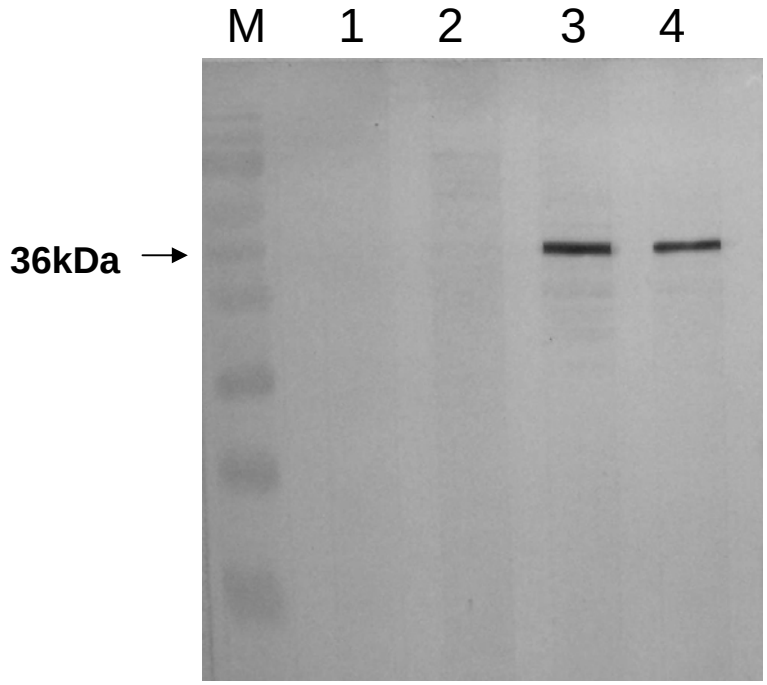
圖二十　大腸桿菌表現系統所得之重組過敏原 rFor t 1 與台灣鋏蠓粗粹物經 SDS-PAGE 與以病患血清做免疫轉漬法。M 欄為 molecular marker；A 欄為尚未經 IPTG 誘發之培養液之 SDS-PAGE；B 欄為經 0.1mM IPTG 誘發培養液之 SDS-PAGE，可得一 24kDa 之融合重組過敏原蛋白；C 欄為台灣鋏蠓粗粹物經 SDS-PAGE 後再與病患血清做免疫轉漬;D 欄為 rFor t 1 經 SDS-PAGE 後再與病患血清做免疫轉漬。



圖二十一　以 ELISA 測定 rFor t 1 之 IgE 活性。第 1、2 為無台灣銨蠓過敏之對照組(NAC)，第 3 至 30 欄為為台灣銨蠓過敏者之血清。28 名台灣銨蠓過敏者中，19 名病患(67.8%)之血清與 rFor t 1 之反應 O.D.值在 cutoff value 以上，呈陽性反應。



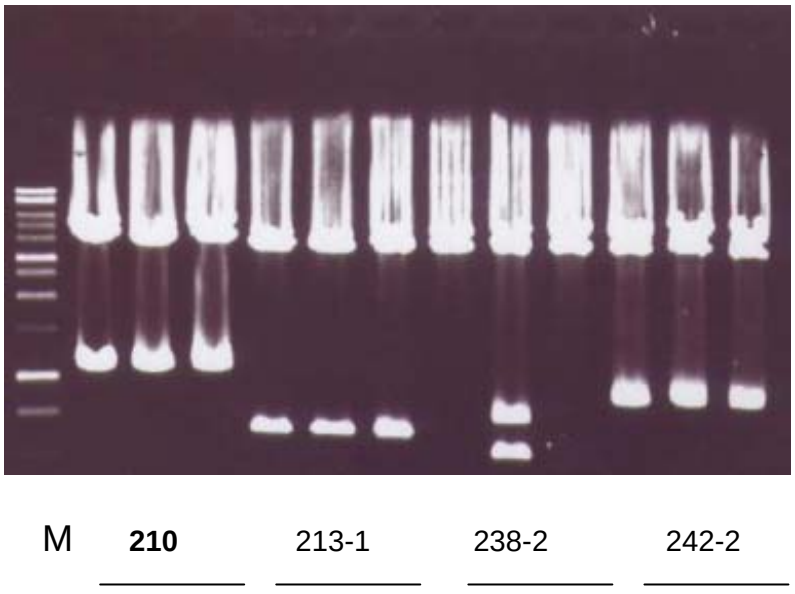
圖二十二　以免疫轉漬法確認 anti-For t 2 抗體之專一性。以 For t 2 守恒域(conserve domain) ^{183}IVPIVEPEVLPDGEHDLDR^{201} 胜肽於兩隻紐西蘭白兔得到之 IgG 抗體，與台灣鋏蠓粗粹物做免疫轉漬法，與於 36kDa 之台灣鋏蠓蛋白質有專一性之結合(如箭號)，與預期辨認之 36kDa For t 2 過敏原位置相符。



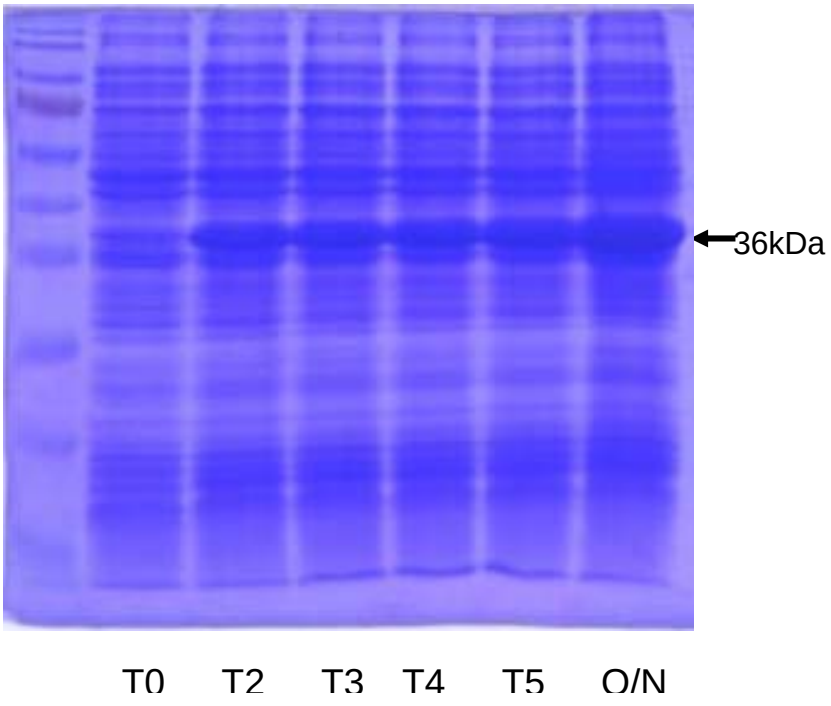
Marker: 100, 72, 55, 40, 33, 24, 17, 11 kDa
Lane 1: Pre-serum R1
2: Pre-serum R2
3: Rabbit 1 anti-For t 2 (1:1000)
4: Rabbit 2 anti- For t2 (1:1000)

圖二十三　與 anti-For t 2 抗體反應呈陽性選株之以 T3 及 T7 引子將 IgE binding clone 做 PCR 增幅(A)；(B)以 0.05mM IPTG 誘發重組蛋白質之產生，T0 表 0 小時，T2 表 2 小時，T3 表 3 小時，T4 表 4 小時，T5 表 5 小時，O/N 表反應隔夜。

(A)



B



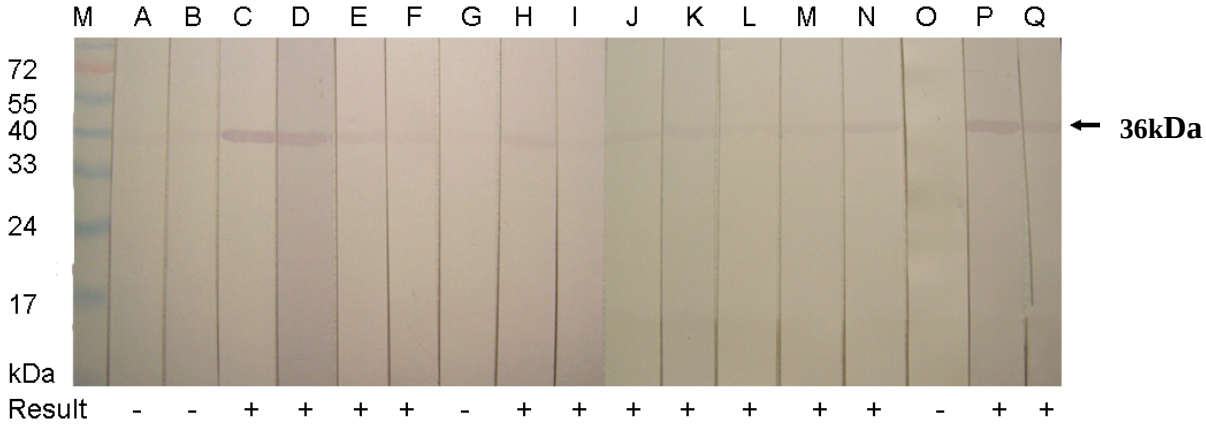
圖二十四　選株 TF210 之核苷酸與胺基酸序列。編號 TF210 之選株經核苷酸定序，共有 1065 個核苷酸，為一 full clone，15-992 為 coding region，其 open reading frame 有 978 個核苷酸，經轉譯後有 325 個胺基酸。其換算預估之蛋白質之分子量為 36kDa，等電點為 5.28。經向 WHO/IUIS 登錄，選株 TF210 正式命名為 For t 2.0101 。

```
GGCACGAGGCAAATATGAAACCTCTGATGATGCAGGGGCACGAGCGTGCCATCACGCAGATC   62
                M  K  P  L  M  M  Q  G  H  E  R  A  I  T  Q  I    16
AAATACAACCGGGAAGGAGACCTCCTGTTCTCGTGCGCGAAGGATCACAAGCCCAACGTCTGG  125
K  Y  N  R  E  G  D  L  L  F  S  C  A  K  D  H  K  P  N  V  W    37
TTTTCGCTGAATGGGGAGCGTTTGGGGACCTTCAATGGACACGCCGGTGCCGTATGGTGCGTC  188
F  S  L  N  G  E  R  L  G  T  F  N  G  H  A  G  A  V  W  C  V    58
GACGTCGACTGGACGACCACGAAGCTCATCACCGGCAGCGGAGACATGTCGGTTCGTCTGTGG  251
D  V  D  W  T  T  T  K  L  I  T  G  S  G  D  M  S  V  R  L  W    79
GACGTCGAGACCGGCACCTCTGTCGCCTGCATTCCGTGCAAATCCTCCGCCAGGACGGTTGGG  314
D  V  E  T  G  T  S  V  A  C  I  P  C  K  S  S  A  R  T  V  G   100
TTCAGCTTCTCCGGCAACCAGGCGGCCTACTCGACAGATCGGGCCATGGGTCACATCTGCGAG  377
F  S  F  S  G  N  Q  A  A  Y  S  T  D  R  A  M  G  H  I  C  E   121
CTCTTCGTTATCGACAGCCGGGAGCCGGATAGCTCTCTCAACGACGGTGACGCGATCCTGAAG  440
L  F  V  I  D  S  R  E  P  D  S  S  L  N  D  G  D  A  I  L  K   142
ATCCCCATCAGCCAGTCAAAGATCACGGCTATGATTTGGGGCACTCTGGACGAGACCATCATC  503
I  P  I  S  Q  S  K  I  T  A  M  I  W  G  T  L  D  E  T  I  I   163
ACGGGTCACGAGAACGGACAGATCACGCTGTGGGACCTCCGCATAGGGCGCGAGATCAACTCC  566
T  G  H  E  N  G  Q  I  T  L  W  D  L  R  I  G  R  E  I  N  S   184
GTGAATGACCACATGGGAGCCATCAACGACCTCCAGCTGTCGAAGGACGGAACCATGTTCGTA  629
V  N  D  H  M  G  A  I  N  D  L  Q  L  S  K  D  G  T  M  F  V   205
TCCTCGTCCCGCGACACGACCGCCAAGCTTTTCGACTCGGACTCGCTCATGTGCTTGAAGACA  692
S  S  S  R  D  T  T  A  K  L  F  D  S  D  S  L  M  C  L  K  T   226
TACAAGACCGAGAGACCCGTCAACTCGGCCGCCATCAGCCCCTTGCTGGAGCACGTGGTGCTA  755
Y  K  T  E  R  P  V  N  S  A  A  I  S  P  L  L  E  H  V  V  L   247
GGAGGAGGACAGGACGCCATGGAGGTCACGACGACGTCCGCGCGCCAGGGCAAGTTCGACTCT  818
G  G  G  Q  D  A  M  E  V  T  T  T  S  A  R  Q  G  K  F  D  S   268
CGCTTCTTCCATCTCATCTACGAGGAGGAGTTTGCTCGCGTGAAGGGCCATTTCGGTCCCATC  881
R  F  F  H  L  I  Y  E  E  E  F  A  R  V  K  G  H  F  G  P  I   289
AACAGTCTGGCCTTCCACCCCGACGGCAGGAGCTACGCCACCGGCGGAGAGGACGGTTTCGTG  944
N  S  L  A  F  H  P  D  G  R  S  Y  A  T  G  G  E  D  G  F  V   310
CGGTTACAGACCTTCGACTCCAGCTACTACGAGTACATCTTTGACTAAGCCGAACTGTATTGG 1007
R  L  Q  T  F  D  S  S  Y  Y  E  Y  I  F  D  *                 325

GGAATTTTGGAATAAACTGGAAGAAAAACGCACGTGCTGTCAAAAAAAAAAAAAAAAAA    1066
```

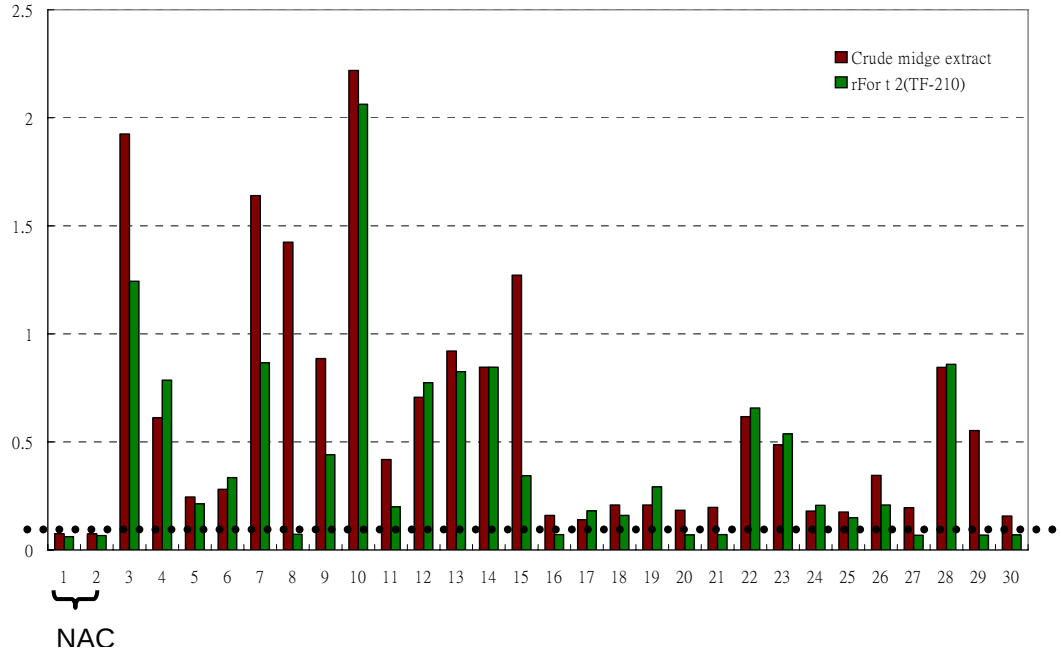
圖二十五　過敏原 For t 2.0101 經 NCBI blastp 比對結果，此蛋白質與埃及斑蚊之 eukaryotic translation initiation factor 3 subunit (eIF3)有77.9%之序列相似性。

```
             10        20        30        40        50        60
For    MKPLMMQGHERAITQIKYNREGDLLFSCAKDHKPNVWFSLNGERLGTFNGHAGAVWCVDV
       ::::::::::::::::::::::::.:: :::::::.:::::::::::::::::: ::::::::
Aed    MKPLMLQGHERAITQIKYNREGDLIFSTAKDHKPSVWFSLNGERLGTFNGHQGAVWCVDV
             10        20        30        40        50        60

             70        80        90       100       110       120
For    DWTTTKLITGSGDMSVRLWDVETGTSVACIPCKSSARTVGFSFSGNQAAYSTDRAMGHIC
       ::::::::::::::..:::::::: .. :::.:.::::.::.::::::.::::.:::: :
Aed    DWTTTRLITGSGDMSTKLWDVETGSVLGTIPCNSAARTVNFSYSGNQASYSTDKAMGHNC
             70        80        90       100       110       120

            130       140       150       160       170
For    ELFVIDSREPDSSLNDGDAILKIPISQ-SKITAMIWGTLDETIITGHENGQITLWDLRIG
       :::.:: :. :::... . .::. ..: ::::.:.::.::::.::::::::.: .:::: .
Aed    ELFIIDVRNVDSSISNQSPVLKLTMNQQSKITSMLWGALDETVITGHENGSIRIWDLRAA
            130       140       150       160       170       180

    180       190       200       210       220       230
For    REINSVNDHMGAINDLQLSKDGTMFVSSSRDTTAKLFDSDSLMCLKTYKTERPVNSAAIS
       .:.:::::::   :::.:::.::::.::.:.:.::::::::.:::::::::::::::::::
Aed    KELNSVNDHTLNINDMQLSSDGTMLVSASKDATAKLFDSESLMCLKTYKTERPVNSAAIS
            190       200       210       220       230       240

    240       250       260       270       280       290
For    PLLEHVVLGGGQDAMEVTTTSARQGKFDSRFFHLIYEEEFARVKGHFGPINSLAFHPDGR
       :. :::::::::.:::::::.. :::::::::.:::::::::::::::::::::::::::.
Aed    PIHEHVVLGGGQEAMEVTTTSTKAGKFDSRFFHLVYEEEFARVKGHFGPINSLAFHPDGK
            250       260       270       280       290       300

    300       310       320
For    SYATGGEDGFVRLQTFDSSYYEYIFD
       ::::::::::::.:.::.:::...:.
Aed    SYATGGEDGFVRVQVFDASYYDFVFE
            310       320
```

圖二十六　以免疫轉漬法測定重組過敏原 rFor t 2.0101 之與 17 名立即型台灣鋏蠓過敏病患血清 IgE 結合活性。其中 13 名病患之血清(病患 C、D、E、F、H、I、J、K、L、M、N、P、Q)可與 rFor t 2.0101 反應，佔 76.4%。



圖二十七　以酵素聯結免疫吸附法(ELISA)分析重組過敏原之 IgE 活性。28 名台灣鈸蠓過敏者中，21 名病患(75%)之血清與 rFor t 2.0101 反應之 O.D.值在 cutoff value 以上，呈陽性反應。NAC 為無台灣鈸蠓過敏之對照組受試者血清。



附　錄

附錄 1. 溶液配製

以下溶液於實驗室中配製, 配製方法如下:

- Phosphate-buffered Saline（PBS）：溶解 8.0 公克 NaCl、0.2 公克 KCl, 1.44 公克 Na_2HPO_4、0.1 公克 Thimerosal 及 0.24 公克 KH_2PO_4 在 800 毫升蒸餾水中。 調整 pH 至 7.2-7.4， 調整體積至 1 公升。以壓力鍋高壓滅菌。

- Tris-buffered Saline（TBS）：溶解 8.76 公克 NaCl 及 2.42 公克 Tris base 於 800 毫升蒸餾水。. 以 1M HCl 調整 pH 至 7.4 。調整體積至 1 公升。以壓力鍋高壓滅菌。

- TBST：溶解 8.76 公克 NaCl、 2.42 公克 Tris base 及 500 微升 Tween-20 於 800 毫升蒸餾水。. 以 1M HCl 調整 pH 至 7.5 。調整體積至 1 公升。以壓力鍋高壓滅菌。

- 2％ Bovine Serum Albumin in TBS：加 2 公克 of bovine serum albumin 至 100 毫升 TBS，使完全溶解，以 NaOH 調整 pH 至 7.4。
- 30％ Acrylamide/Bis- acrylamide：溶解 29.2 公克 acrylamide 及 0.8 公克 N,N’-methylene-bis-acrylamide 於 80 毫升蒸餾水。調整體積

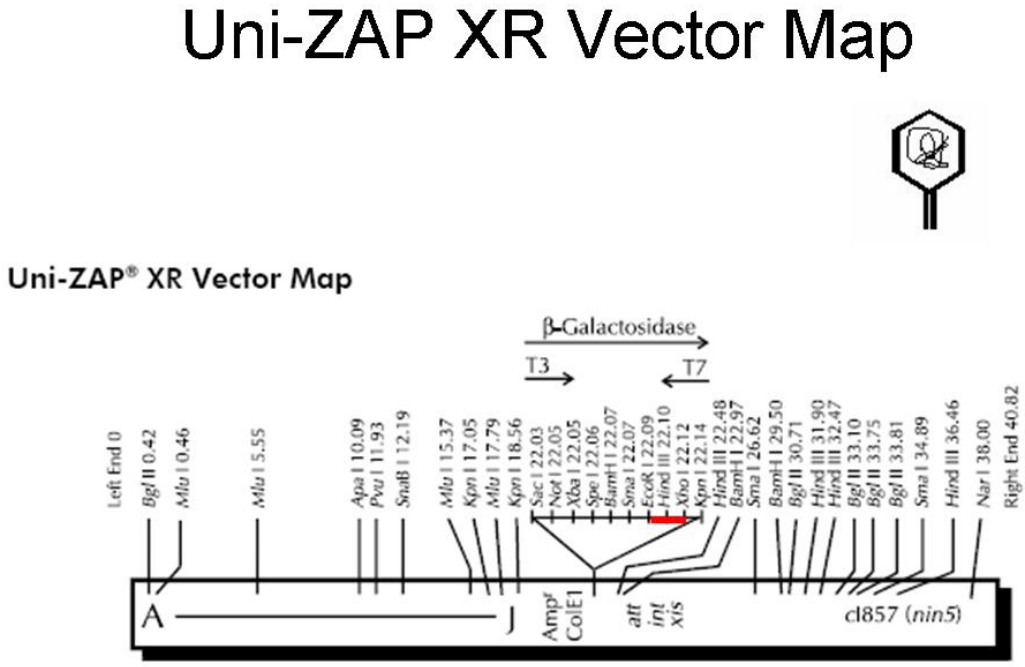
至 100 毫升。

- 1.5 M Tris（pH 8.8）：溶解 181.71 公克 Tris base 於 800 毫升蒸餾水。以濃縮 HCl 調整 pH 至 8.8 。調整體積 to 1 公升。.
- 0.5 M Tris（pH 6.8）：溶解 6 公克 Tris base 於 80 毫升蒸餾水。以濃縮 HCl 調整 pH 至 6.8。調整體積至 100 毫升。
- 10% Sodium Dodecyl Sulfate（SDS）：溶解 10 公克 SDS 於 100 毫升蒸餾水，使完全溶解。
- 10x Laemmli Running Buffer：加 8 公升蒸餾水於十公升容器中，加 303 公克 Tris base, 1442 公克 glycine, 及 100 公克 SDS，以蒸餾水調整體積至 10 公升。
- Coomassie blue G-250 staining solution： 將 0.5 公克 Coomassie blue G-250 染劑溶於 200 毫升 acetic acid 加 800 毫升蒸餾水，攪伴一小時後，以 Whatman 一號濾紙過濾。
- ELISA coating buffer：溶解 5.3 公克 Na_2CO_3 、4.2g $NaHCO_3$ 、1g sodium azide 於 900 毫升蒸餾水，調 pH 至 9.6，以蒸餾水調體積至 1 公升。
- 4% SDS-PAGE stacking gel：1.3 毫升 30% Acrylamide/Bis(29.2:0.8)+ 6.1 毫升蒸餾水+2.5 毫升 0.5M Tris-HCL, pH6.8+100 微升 10% SDS+100 微升 10% APS(freshly prepared)+10

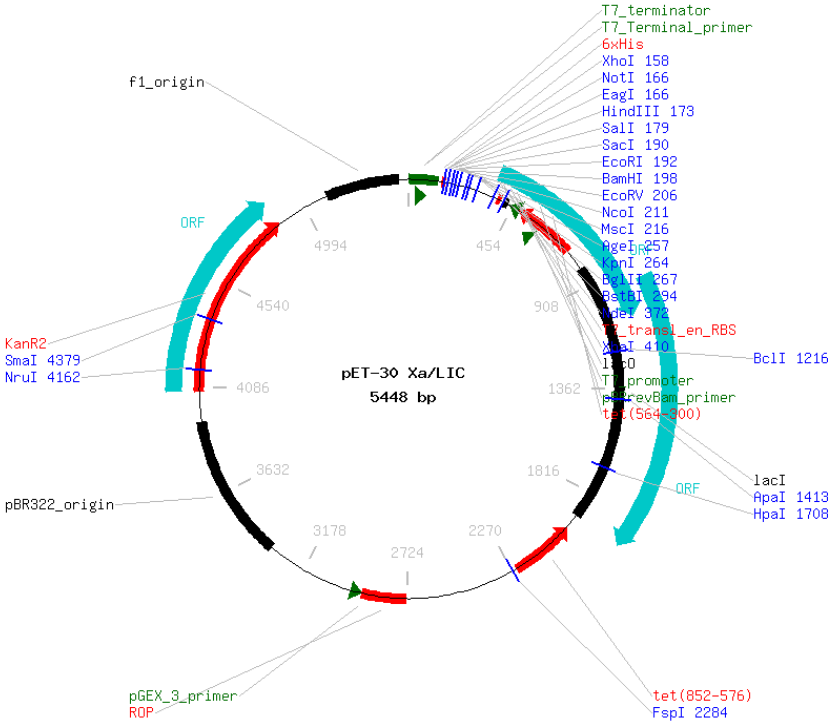
微升 TEMED

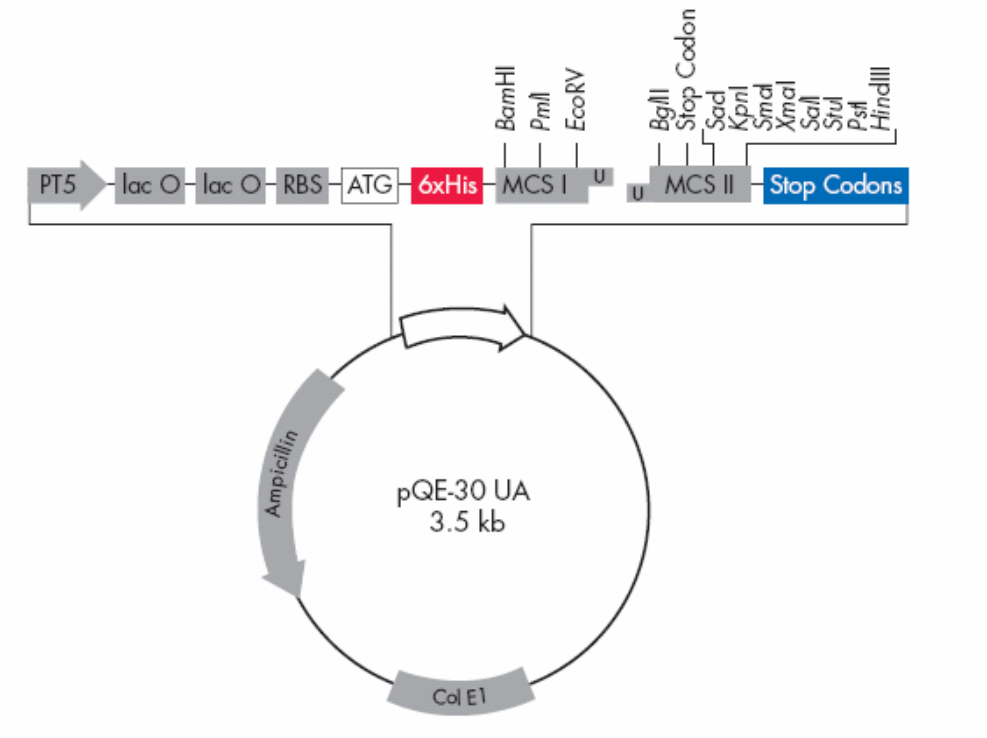
- 12% SDS-PAGE separating gel：8 毫升 30% Acrylamide/Bis(29.2:0.8)+ 6.7 毫升蒸餾水+5 毫升 1.5M Tris-HCL, pH8.8+200 微升 10% SDS+100 微升 10% APS(freshly prepared)+10 微升 TEMED

附錄 2 ZAP 載體之載體地圖。紅線為 cDNA insert 之位置。



附錄 3 pET30 與 pQE30 載體之載體地圖





附錄 4 台中榮民總醫院人體試驗委員會許可書

IRB TCVGH

The Institutional Review Board of Taichung Veterans General Hospital

台中榮民總醫院人體試驗委員會

Taichung Veterans General Hospital, Taichung, Taiwan 40705, ROC
TEL:886 4-23592525-4006 FAX:886-4-23592705
E-mail: irb@ vghtc.gov.tw

人體試驗研究計畫許可書

中華民國 93 年 1 月 19 日

計畫名稱：台灣鈇蠓過敏原基因表現、純化、免疫特性及臨床表徵之研究

研究計畫主持人：免疫風濕科陳怡行醫師 (本會編號：921218/271)

上述計畫已於中華民國 93 年 1 月 5 日經本院人體試驗委員會第三十六次會議審查通過。本證明有效期限從 93 年 8 月 1 日至 94 年 7 月 31 日止。

人體試驗委員會主任委員

副院長 陳穎從

陳穎從

Permission of Clinical Trial
Taichung Veterans General Hospital

Date. 19 January 2004

The project entitled" Expression, Purification, Characterization and Clinical Relevance of *Forcipomyia Taiwana* Allergy in Taiwan" (*IRB* TCVGH No : 921218/271) submitted by the investigator Yi-Hsing Chen, was approved by the Institutional Review Board of Taichung Veterans General Hospital at the 36th committee meeting on 5 January 2004. This permission is valid from 1 August 2004 to 31 July 2005.

Ying-Tsung Chen

Chairman, Ying-Tsung Chen, M.D.
Institutional Review Board

本會組織與執行皆符合 ICH-GCP
The committee is organized and operates according to GCP and the applicable laws and regulations

IRB TCVGH

The Institutional Review Board of Taichung Veterans General Hospital

台中榮民總醫院人體試驗委員會

Taichung Veterans General Hospital, Taichung, Taiwan 40705, ROC

TEL:886-4-23592525-4006 FAX:886-4-23592705

E-mail: irb@ vghtc.gov.tw

人體試驗研究計畫許可書

中華民國 95 年 1 月 19 日

計畫名稱：以DNA重組技術建立台灣蛺蠓(小黑蚊)過敏之標準常規檢驗法

計畫主持人：過敏免疫風濕科陳怡行醫師(本會編號：941222/ C05214)

共同主持人：教學研究部蔡肇基主任及李美芳助理研究員

上述計畫於中華民國95年1月19日經本院人體試驗委員會快速審查通過，將於本會第58次會議追認。本證明有效期限至96年1月18日止。

人體試驗委員會主任委員

副院長　陳穎從

Permission of Clinical Trial
Taichung Veterans General Hospital

Date: 19 January 2006

The project entitled, " Development of standard routine diagnostic methods of *Forcipomyia taiwana* allergy using recombinant DNA technology " (*IRB* TCVGH No : 941222/ C05214) submitted by the investigator Yi-Hsing Chen, the co-investigators Jaw-Ji Tsai, and Mey-Fann Lee, was approved by the Institutional Review Board of Taichung Veterans General Hospital on 19 January 2006 by the expedited review procedure. The approval will be reported at the 58th full committee meeting of the IRB. This permission is valid to January 18, 2007.

Ying-Tsung Chen, M.D.

Chairman, Institutional Review Board, TCVGH

人體試驗委員會 台中榮民總醫院

本會組織與執行皆符合 ICH-GCP

The committee is organized and operates according to GCP and the applicable laws and regulations

附錄 5 已發表之期刊論文(一)

Y. H. Chen, M. F. Lee, J. L. Lan, C.S.Chen, H.L. Lin, G. Y. Huang, and C. H. Wu*. Hypersensitivity to *Forcipomyia taiwana* (biting midge): Clinical analysis and identification of major For t 1, For t 2, For t 3 allergens. *Allergy* .2005 Dec;60(12):1518-23 (SCI＝5.014)

附錄 6 已發表之期刊論文（二）

Y.H. Chen, M.F. Lee, J.J. Tsai, H.J. Wu, G..Y. Hwang*. Specific IgE and IgG responses and cytokine profile in subjects with allergic reactions to biting midge *Forcipomyia taiwana*. *Int Arch Allergy Immunol.* 2009 Apr; 150(1):66-74 (SCI=2.16)

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ALLERGY
DOI: 10.1111/j.1398-9995.2005.00918.x

Original article

Hypersensitivity to *Forcipomyia taiwana* (biting midge): clinical analysis and identification of major For t 1, For t 2 and For t 3 allergens

Y.-H. Chen[1,3], M.-F. Lee[2,3], J.-L. Lan[1], C.-S. Chen[3], H.-L. Wang[2], G.-Y. Hwang[3], C.-H. Wu[2]

[1]Department of Internal Medicine, Division of Allergy, Immunology and Rheumatology; [2]Department of Education and Research, Taichung Veterans General Hospital; [3]Department of Life Science, Tunghai University, Taichung, Taiwan

Background: *Forcipomyia taiwana* is a tiny, blood-sucking midge that cause intense pruritis and swelling in sensitive individuals. It is distributed island-wide in rural Taiwan and Southern China.
Objective: This study aimed to study the allergic immune responses and identify *F. taiwana* allergens.
Methods: Crude whole body *F. taiwana* extracts were prepared with phosphate-buffered saline. The specific IgE antibody was determined by enzyme-linked immunoassay and immunoblotting. Protein was analyzed by electrospray ionization tandem mass spectrometry.
Results: Among the 372 subjects that were exposed to *F. taiwana* bites, 179 (48%) reported an immediate skin reaction with/without delay reaction and 41(11.1%) reported a solely delay reaction. The skin of 21 subjects was tested with *F. taiwana* extract. Of these 21 subjects, 12 (57.1%) produced immediate skin reactions and contained high levels of specific IgE antibody against *F. taiwana*. Immunoblotting revealed that 11 allergenic components are able to bind specific IgE. Allergens of 22, 24, 35, 36, and 64 kDa bound 50, 50, 75, 66.7, and 75% of IgE-containing sera tested, respectively. Tryptic fragments of the 24, 35, 36, and 64 kDa allergens were analyzed by ESI-MS/MS. Selected tryptic peptides of 24, 35, and 36, and 64 kDa allergens exhibited significant sequence identity with triosephosphate isomerase of *Anopheles merus, Tenebrio molitor, Ochlerotatus togoi,* and *Chrysops vittatus,* fructose 1,6-bisphosphate aldolase of *Antheraea yamamai* and *Homalodisca coagulata,* and a slow muscle myosin S_1 heavy chain of *Homarus americanus* and a protein with unknown function from *A. gambiae,* respectively. The 35 and 36 kDa proteins may represent different isoforms of the fructose 1,6-bisphosphate aldolase.
Conclusion: We conclude that immediate reaction to *F. taiwana* bites is IgE mediated and the 24 (For t 1), 35 (For t 2), and 64 kDa (For t 3) proteins are candidates for major *F. taiwana* allergens. Further studies are needed to confirm these allergens.

Key words: allergy; biting midge; *Forcipomyia taiwana*; insect allergens.

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Accepted for publication 3 May 2005

Insects have been documented to be an important source of allergens in humans since antiquity. The body components of insects can become inhalant allergens causing allergic rhino-conjunctivitis and asthma. Stinging insects can cause allergic reactions by directly introducing saliva or venom into human skin (1, 2). The allergic reactions of stinging insects range from pruritic local reactions to fatal anaphylactic shock and continue to be a serious medical problem (3, 4).

Shiraki first reported the presence of *Forcipomyia* (Lasiohelea) *taiwana* (Shiraki) in central Taiwan in 1913 (5). *Forcipomyia taiwana* is a tiny (1–1.5 mm) blood-sucking midge that is distributed island-wide in urban and suburban Taiwan as well as in Southern China. Its larvae live in moderately moist soil around housing or shaded areas of cultivated bamboo, tea, vegetable and betel nuts (6–8). Female *F. taiwana* midges attack exposed parts of the body during the day, causing intense pruritis and swelling in sensitive individuals. Following an outbreak in 1989, it spread rapidly and became abundant in certain areas of Taiwan, such as the Ta-Ken foothill area of Taichung, and has become one of the most abundant bloodsucking pests in Taiwan. Although local physicians have been aware of severe reactions to *F. taiwana* bites for a long time, there are no clinical data regarding *F. taiwana* allergy, and the antigenic and allergenic properties of *F. taiwana* have never been documented. In the present study, we report the immune responses to *F. taiwana* and the identification of 24, 35, and 64 kDa major allergens of *F. taiwana*. The 24, 35, and 64 kDa proteins, according to World Health

Organization (WHO) and the International Union of Immunological Societies (IUIS) nomenclature, are designated as For t 1, For t 2, and For t 3, respectively.

Methods

Subjects

During a two 5-month periods (May 2003–September 2003, and May 2004–September 2004), 372 subjects with history of *F. taiwana* bite were selected from the allergy clinic at Taichung Veterans General Hospital and a college in Ta-Ken, Taichung, Taiwan and interviewed by a nurse using a questionnaire regarding exposure and detailed reactions to *F. taiwana*. Reactions developed within 1 h of bites are defined as immediate reaction and that developed after 6 h or more as delayed reaction. Those presented both immediate and delay reactions were categorized into the immediate reaction group.

Preparation of whole body *F. taiwana* extract

Female midges were collected by human bait method using special collectors designed by Dr C. S. Chen's laboratory, Department of Life Science, Tunghai University, Taichung, Taiwan. One thousand females midges were ground and dissolved in 5 ml of phosphate buffered saline (PBS), ultrasonicated for 30 min at 4°C, and centrifuged at 8000 *g* for 15 min. The supernatant was collected, filtered through 0.45 μm filter, aliquoted, and stored at −70°C.

Skin testing and patients' sera

Crude *F. taiwana* extract at a concentration of 200 μg/ml in PBS containing 50% glycerol was used for skin testing using epicutaneous sterile disposable Sharp Test® applicators (Greer Laboratories, Lenoir, NC). Histamine (1 mg/ml) and 50% PBS-glycerol were used as positive and negative controls, respectively. All skin test results were read 20 min after placement. A response with a wheal or an erythema 3 mm larger in size than that produced by the negative control but less than 1/2 of the diameter of the positive control was graded as 1+, and a reaction ranging between 1/2 and equivalent size of the positive control was graded as 2+. A reaction compatible with the positive control was graded as 3+. A reaction larger than the positive control was grade as 4+. Sera were collected after skin test.

Allergen specific IgE

Optimal concentrations of antigen and conjugate were determined by checkerboard titration (9), and level of IgE specific to crude *F. taiwana* extract (3.0 μg/well) was measured using a commercial enzyme allergosorbent test (EAST) kit according to the manufacturer's instructions (Allergopharma, Reinbek, Germany). Reference disc coated with *Phleum pratense* allergen and reference sera containing 17.5, 3.5, 0.7, and 0.35 kU/l were used as positive controls, and sera from nonallergic subjects were used as negative controls.

Western blot analysis

Crude *F. taiwana* extract was subjected to standard SDS-PAGE under denatured conditions with a discontinuous buffer, and proteins were transferred onto PVDF membranes and immunodetected according to the method previously described (10).

Two-dimensional gel electrophoresis (2-D)

Crude *F. taiwana* extract was subjected to 2-D under denatured conditions. The first dimension of 2-D was performed with a Bio-Rad Protean II xi 2-D cell system (Bio-Rad) with pH gradient (Bio-Rad) from 3 to 10 for a total volt-hour of 14 200. The second dimension SDS-PAGE was then performed according to the methods recommended by the manufacturer and immunoblotting was performed.

Mass spectrometry

Proteins were visualized with silver stain after 2-D, and the target protein spots were excised for in-gel digestion using a sequencing-grade modified trypsin (Promega, Madison, WI). The tryptic peptide mixtures were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS; Finnigan MAT, San Jose, CA, USA) and searched with a Mascot computer program (Matrix Science).

Results

Incidence and manifestations of hypersensitive reactions to *F. taiwana*

A total of 372 (153 males and 219 females) subjects who had been exposed to *F. taiwana* (Fig. 1) bites were interviewed by questionnaire. The mean age of subjects was 38.59 ± 15.25 years, with a range of 1–80 years. Of the 372 subjects, 59.1% (220/372) were found to have immediate and/or delay type reactions to *F. taiwana*. There were no significant differences in incidence of *F. taiwana* allergy with regard to sex, age or concomitant atopic diseases (Table 1). Large wheals developed within 1 h of bite, and the size of swelling varied from 2 cm to more than 10 cm (Fig. 2A). In some patients, delayed papular-vesicles developed following immediate reaction (Fig. 2B). In the solely delayed reactions, itching papules

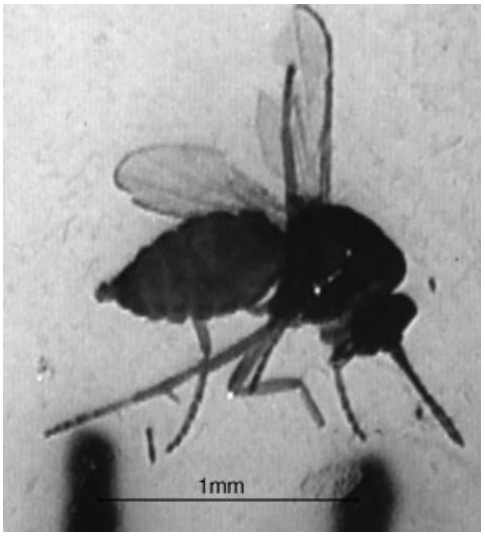

Figure 1. Picture of *Forcipomyia taiwana,* under 10× microscope.

or vesicles developed in 6–24 h, and the intense pruritic lesions become centrally necrotic later in some subjects (Fig. 2C). The delayed skin lesions persisted for weeks, or even months in some individuals.

Allergy skin test and IgE antibodies

Twenty-one subjects with a history of hypersensitive reaction to *F. taiwana* bites were randomly selected and skin tested with *F. taiwana* extract after informed consent. Ten individuals with no reaction to *F. taiwana* bites showed negative skin reaction and no specific IgE antibody against *F. taiwana*. Of the 21 patients, 57.1% (12/21) gave a positive immediate skin reaction and 42.9% (10/21) gave a solely delayed reaction. IgE antibody for patients with immediate and solely delayed reactions gave a mean value of 17.75 ± 6.72 and 3.58 ± 2.84 kU/l, respectively, as defined by EAST. The results are summarized in Table 2.

SDS-PAGE and immunoblotting

Resolution of proteins of *F. taiwana* by SDS-PAGE revealed at least 33 proteins, with molecular weights (MW) ranging from 10 to 70 kDa, that were visible following silver staining (data not shown). Immunoblotting revealed that 11 allergenic components are able to bind specific IgE (Fig. 3, lanes a–l). Allergens of 10, 20, 22, 24, 29, 30, 31, 35, 36, 37, and 64 kDa bound 8.3% (1/12), 33.3% (4/12), 50% (6/12), 50% (6/12), 33.3% (4/12), 116.7% (2/12), 33.3% (4/12), 75% (9/12), 66.7% (8/12), 8.3% (1/12), and 75% (9/12) of the sera tested, respectively. Except for nonspecific binding to a band of around 29 kDa found in all of the sera, sera from *F. taiwana*-negative atopic and nonatopic subjects (Fig. 3, lanes m and n) and patients with solely delayed skin reaction (patients 13–21, data not shown) did not display any IgE binding to any *F. taiwana* proteins.

Table 1. Incidence of hypersensitive reactions to *F. taiwana*

Reactions	No. of subjects ($n = 372$)	Percent (%)
None	152	40.9
Immediate reaction	179	48.0
Solely delay reaction		
Papules	20	5.4
Vesicles	13	3.5
Lymphadenopathy	1	0.3
Fever	3	0.8
Others	4	1.1

The 2-D and ESI-MS/MS

The isoelectric points (pI) of the 24 (For t 1), 35, 36, and 64 kDa (For t 3) *F. taiwana* allergens were 5.8, 8.6, 8.7, and 5.2, respectively as revealed by 2-D (data not shown). The stable signals obtained from tryptic peptide mixtures of the For t 1, 35, 36 kDa and For t 3 allergens

Table 2. Clinical characteristics of hypersensitive reactions to *F. taiwana*

Subject	Age	Sex	Symptoms	Skin test/kU/L	Atopy	Autoimmune disease
1	42	F	I, D	4+/16.39	AR	–
2	23	F	I, D	1+/9.28	–	SLE
3	28	M	I, D	4+/22.89	–	–
4	37	M	I	3+/11.94	–	–
5	14	M	I, D	2+/16.43	AR	–
6	24	F	I, D	1+/8.25	–	–
7	53	M	I, D	4+/10.56	–	SLE
8	39	F	I, D	3+/19.34	–	–
9	18	M	I	2+/21.9	AR, AS, AD	–
10	37	F	I, D	1+/22.56	–	–
11	29	F	I, D	2+/29.98	–	SLE
12	60	F	I	2+/11.5	–	–
13	45	F	D	–/0.65	–	–
14	24	F	D	–/8.25	–	–
15	36	F	D	–/1.40	AR	–
16	50	F	D	–/3.04	AR	–
17	29	M	D	–/6.78	–	–
18	45	F	D	–/0.72	–	–
19	22	F	D	–/5.32	AR	–
20	37	F	D	–/4.94	–	–
21	24	F	D	–/1.12	AR	–

I, immediate reaction; D, delayed reaction; AS, asthma; AR, allergic rhinoconjunctivitis; AD, atopic dermatitis; SLE, systemic lupus erythematous.

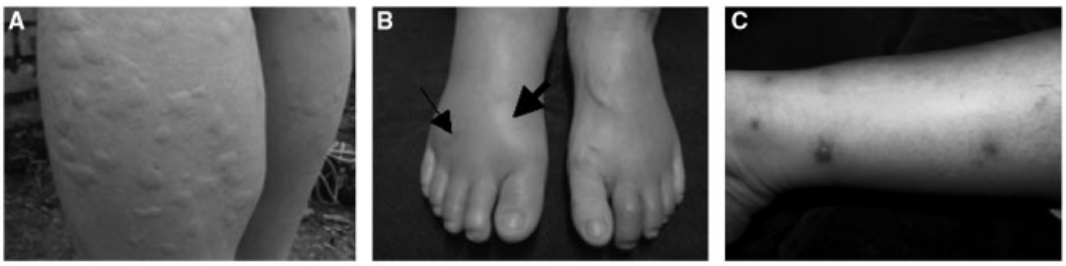

Figure 2. (A) Immediate reaction to *Forcipamyia taiwana* bites. Large wheals usually develop within 1 h of bites and the sizes vary from 2 cm to more than 10 cm. (B) Immediate reaction plus delayed papular-vesicles. Note the large local swelling at right foot designated by thick arrow and small vesicles designated by thin arrow. (C) Solely delayed reactions. Note the erythematous papules and vesicles over the lower leg of the patient.

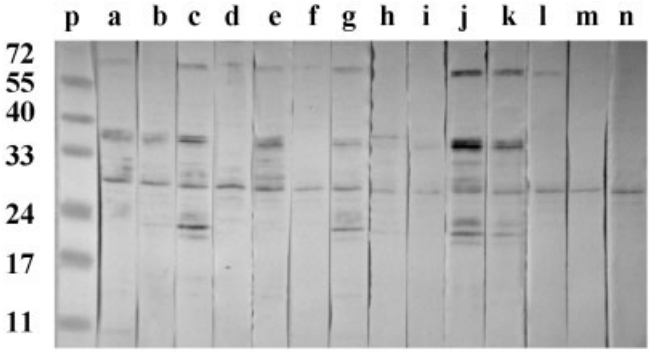

Figure 3. Immunoblot patterns of crude *F. taiwana* extract with immediate reaction to *F. taiwana* subjects 1–12 (lanes a–l), *F. taiwana*-negative atopic (lane m) and nonatopic (lane n) subjects. Numbers at left indicate size of standard proteins in kDa (lane p).

were analyzed by ESI-MS/MS and identified as triosephosphate isomerase (TPI) of *Anopheles merus* (fly), *Tenebrio molitor* (yellow mealworm), *Ochlerotatus togoi* (fly) and *Chrysops vittatus* (deer fly), fructose 1,6-bisphosphate aldolase of *Homalodisca coagulata* (glassy-winged sharpshooter) and *Antheraea yamamai* (Japanese oak silkmoth) and a slow muscle myosin S_1 heavy chain of *Homarus americanus* (American lobster), respectively (Table 3). The 35, 36 kDa (For t 2) proteins may represent different isoforms of the fructose 1,6-bisphosphate aldolase. Moreover, using the same technique, 13 tryptic peptides obtained from the For t 3 allergen, showed sequence identity with an unpublished protein sequence of the African malaria mosquito (*A. gambiae* str. PEST; 151.6 kDa, pI 5.57; GenBank gi:31235885).

Discussion

Seasonal allergic dermatitis caused by biting midges in horses, sheep, cattle, and donkey (11–14), have been documented, but clinical manifestations and identification of allergenic components of *F. taiwana* have not been reported in humans. Here we found that 220 out of 372 subjects (59.1%) reported reactions to *F. taiwana* bites. Among these 220 subjects, 179 (81.4%) had immediate large local reactions or immediate large local reactions followed by delayed reactions to *F. taiwana* bites, whereas 33 subjects (15.0%) had delayed cutaneous reactions with either itching papules or vesicles and four subjects (1.8%) had systemic reactions such as fever or lymphadenopathy. Among the 21 selected individuals with history of hypersensitive reaction to *F. taiwana* bites, the mean level for *F. taiwana*-specific IgE was significantly higher (Student's *t*-test, $P < 0.001$) in subjects with immediate reaction than in those with solely delayed reaction. Hypersensitive reactions to mosquito bites are usually IgE-mediated and lymphocyte-mediated (15–17). It is conceivable that the delayed reaction to *F. taiwana* may be caused by IgG or lymphocyte-mediated immune responses.

Triosephosphate isomerase, a glycolytic enzyme, catalyses the isomerization of dihydroxyacetone phosphate into D-glyceraldehyde 3-phosphate, and TPI had been identified as wheat flour (18) and latex (19, 20) allergens. Fructose 1,6-biphosphate (FBP) aldolase (ADL, D-fructose 1,6-biphosphate D-glyceraldehydes 3-*P*-lyase; EC 4.1.2.13) catalyzes the reversible aldol condensation of glyceraldehydes 3-phosphate and dihydroxyacetone phosphate yielding FBP. In class-I aldolase, which is found in animals and higher plants, catalysis depends upon Schiff-base formation with the subtract (21), whereas in class-II aldolase, which is found in fungi and prokaryotes, divalent cations are required for activity (22). Using

Table 3. Identification of *F. taiwana* allergens by ESI-MS/MS

Allergen*	pI*	Matching identity	Species	GenBank access. No.	MW/pI (GenBank)	Matching residue sequence
For t 1 (24 kDa)	5.8	Triosephosphate isomerase (EC 5.3.1.1)	*A. merus*	gi:1857455	21.86/5.33	^{104}VIACIGETLQER^{115} ^{156}TATPEQAQEVHAALRK^{171} ^{187}IQYGGSVTAANCR^{199}
			T. molitor	gi:22090453	26.66/6.0	^{84}DVGADWVILGHSERR^{97} ^{122}VIACIGETLEER^{133} ^{148}AIAAKVNDWSNVVIAYEPVWAIGTGK^{173}
			O. togoi	gi:1857457	21.91/5.30	^{104}VIACIGETLQER^{115} ^{66}DVGANWVIIGHSER^{79}
			C. vittatus	gi:1857453	16.42/5.39	^{171}KWFTDNVSADVSASIRIQYGGSVTAANCR^{199} ^{104}VIACIGETLQER^{115}
For t 2 (35 and 36 kDa)	8.6	Fructose	*H. coagulata*	gi:46561746	39.68/7.56	^{88}ADDGTPFVELLKK^{100} ^{157}NTPSYQAILENANVLAR^{173}
	8.7	1,6-bisphosphate aldolase (EC 4.1.2.13)	*A. yamamai*	gi:45330818	39.67/7.59	^{157}NTPSYQAILENANVLAR^{173} ^{183}IVPIVEPEVLPDGEHDLDR^{201}
For t 3 (64 kDa)	5.2	Slow muscle myosin S1 heavy chain	*H. americanus*	gi:37925239	58.53/5.22	^{16}QRIATELEDMQIEVER^{31} ^{352}AKKAMVDAAR^{361} ^{478}QIEEAEEIAALNLAK^{492}

*Experimental molecular weight and pI.

amino acid sequentiation, ADL (37 kDa) was reported to be a wheat allergen causing bakers' asthma (23, 24). Crustacean muscles are biochemically characterized as fast-twitch, slow-twitch (S_1) and slow-tonic (S_2) types (25). Fiber-type-specific isoforms of myosin heavy and light chains, tropomyosin, paramyosin, and troponin-I, -T and -C are expressed in lobster and crayfish muscles (26). SMM S_1HC from the American lobster was able to bind to the anti-troponin T (TnT) antibody (27), and showed 66% sequence identity with an unknown function protein from the African malaria mosquito. The TnT is a member of the troponin complex, consisting of troponins-I, -C and -T, which is responsible for binding tropomyosin to regulate calcium binding and contractility of vertebrate striated muscles. Tropomyosin is an important allergen found in crustaceans, mollusks and other invertebrates such as lobsters, crabs, shrimps, squids, snails, oysters, house dust mites, and cockroaches (28), and may be the cause of clinical cross-sensitivity among invertebrates (29). These and our results open the possibility that TPI, ADL, and SMMS_1HC may have a tendency to cause allergic diseases and are worthy for further investigation.

In summary, we describe the humoral immune response to *F. taiwana* allergens in selected individuals with immediate and delayed allergic reactions. We identified several allergenic components from the crude extract using sensitive and specific immunoassay. Skin testing revealed that all 12 tested subjects gave a positive reaction, and immunoblotting revealed a prevalence rate of 50, 75, and 75% for For t 1, For t 2, and For t 3 allergens, which indicates that these proteins are candidate as major allergens of *F. taiwana* (at least 50% according to WHO-IUIS). To our knowledge, this is the first study to investigate the antigenic and allergenic properties of *F. taiwana*. Further study is required to characterize and confirm these allergens, to investigate the lymphocyte-mediated reactions to *F. taiwana* and to elucidate the mechanisms of *F. taiwana* hypersensitivity.

Acknowledgments

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References

1. Golden DB. Stinging insect allergy. Am Fam Physician 2003;**67**:2541–2546.
2. Hoffman DR. Allergic Reactions to Biting Insects. In: Levine MI, Lockey RF, editors. Monograph on insect allergy. Milwaukee: American Academy of Allergy Asthma& Immunology, **2003**:161–174.
3. Galindo PA, Gomez E, Borja J, Feo F, Garcia R, Lombardero M et al. Mosquito bite hypersensitivity. Allergol Immunopathol (Madr) 1998;**26**:251–254.
4. Reisman RE. Stinging insect allergy. Med Clin N Am 1992;**76**:883–94.
5. Shiraki T. Investigation on general injurious insect. Taiwan Sotokufu Noji Shikenjo Tokubetsu Hokodu 1913;**8**:286–297.
6. Sun W. Study of a biting midge, *Forcipomyia* (*Lasiohelea*) *taiwana* (Shiraki) (Diptera: Ceratopogonidae) I. Description of the complete life cycle of the midge reared in the laboratory. Biol Bull Tunghai Univ (Taiwan) 1967;**29**:1–10.
7. Chen CS, Lien JC, Lin YN, Hsu SJ. The diurnal biting pattern of a bloodsucking midge *Forcipomyia* (Lasiohelea) *taiwana* (Shiraki) (Diptera, Ceratopogonidae). Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 1981;**14**:54–56.
8. Chuang YY, Lin CS, Wang CH, Yeh CC. Distribution and seasonal occurrence of *Forcipomyia taiwana* (Diptera: Ceratopogonidae) in the Nantou area in Taiwan. J Med Entomol 2000;**37**:205–209.
9. Alsenz J, Loos MA. A rapid and simple ELISA for the determination of duplicate monoclonal antibodies during epitope analysis of antigens and its application to the study of C1-INH. J Immunol Methods 1988;**109**:75–84.
10. Wu CH, Wang NM, Lee MF, Kao CY, Luo SF. Cloning of the American cockroach Cr-PII allergens: evidence for the existence of cross-reactive allergens between species. J All Clin Immunol 1998;**101**:832–840.
11. Yeruham I, Perl S, Braverman Y. Seasonal allergic dermatitis in sheep associated with Ctenocephalides and Culicoides bites. Vet Dermatol 2004;**15**:377–380.
12. Yeruham I, Braverman Y, Orgad U. Field observations in Israel on hypersensitivity in cattle, sheep and donkeys caused by Culicoides. Aust Vet J 1993;**70**:348–352.
13. Anderson GS, Belton P, Kleider N. Hypersensitivity of horses in British Columbia to extracts of native and exotic species of Culicoides (Diptera: Ceratopogonidae). J Med Entomol 1993;**30**:657–663.
14. Wilson AD, Harwood LJ, Bjornsdottir S, Marti E, Day MJ. Detection of IgG and IgE serum antibodies to Culicoides salivary gland antigens in horses with insect dermal hypersensitivity (sweet itch). Equine Vet J 2001;**33**:707–13.
15. Peng Z, Yang M, Simons FE. Measurement of mosquito Aedes vexans salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. Ann All Asthma Immunol 1995;**74**:259–264.
16. Peng Z, Yang M, Simons FE. Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens. Ann All Asthma Immunol 1996;**77**:238–244.
17. Peng Z, Simons FER. Mosquito allergy: immune mechanisms and recombinant salivary allergens. Int Arch All Immunol 2004;**133**:198–209.

18. Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. J All Clin Immunol 2001;**107**:907–913.
19. Posch A, Chen Z, Wheeler C, Dunn MJ, Raulf-Heimsoth M, Baur X. Characterization and identification of latex allergens by two-dimensional electrophoresis and protein microsequencing. J All Clin Immunol 1997;**99**:385–395.
20. Posch A, Chen Z, Dunn MJ, Wheeler CH, Petersen A, Leubner-Metzger G et al. Latex allergens database. Electrophoresis 1997;**18**:2803–2810.
21. Grazi E, Rowley PT, Cheng T, Tchola O, Horecker BL. The mechanism of action of aldolases. III. Schiff base formation with lysine. Biochem Biophys Res Commun 1962;**9**:38–43.
22. Rutter WJ. Evolution of aldolase. Fed Proc 1964;**23**:1248–1257.
23. Weiss W, Huber G, Engel KH, Pethran A, Dunn MJ, Goolev A et al. Identification and characterization of wheat grain albumin/globulin allergens. Electrophoresis 1997;**18**:826–833.
24. Baur X, Posch A. Characterized allergens causing bakers' asthma. Allergy 1998;**53**:562–566.
25. Mykles DL. Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. Comp Biochem Physiol 1997;**117B**:367–378.
26. Mykles DL, Medler S, Koenders A, Cooper R. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J Exp Biol 2002;**205**:513–522.
27. Medler S, Mykles DL. Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster *Homarus americanus*: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres. J Exp Biol 2003;**206**:3557–3567.
28. Ayuso R, Lehrer SB, Reese G. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). Int Arch All Immunol 2002;**127**:27–37.
29. Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch All Immunol 1999;**119**:198–202.

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Specific IgE and IgG Responses and Cytokine Profile in Subjects with Allergic Reactions to Biting Midge *Forcipomyia taiwana*

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Key Words
Bites, insect · Cytokine · *Forcipomyia taiwana* · Immune response · Midge · Protease

Abstract
Background: *Forcipomyia taiwana* is a tiny blood-sucking midge whose habitat covers large parts of Taiwan and southern China. Female midges bite during the day, causing intense pruritis and swelling in allergic individuals. In this study, we investigated the immune responses of different allergic reactions to midge bites. ***Methods:*** *F. taiwana* (midge)-specific IgE, -IgG and -IgG subclasses were examined by ELISA in 62 human subjects. Peripheral blood mononuclear cells (PBMC) from 6 subjects with solely delayed reactions (SDR) to midge bites and 6 nonallergic controls (NAC) were cultured with midge extract at various time points and assayed. Proliferation of PBMC was measured by MTT assay. Expression of cytokine mRNA was measured by real-time PCR and protein levels by cytometric bead immunoassay or ELISA. Protease activity in midge extract was determined by the Azocoll method. ***Results:*** Midge-specific IgE among subjects with an immediate reaction were significantly elevated compared to SDR and NAC subjects. There were no differences in the level of midge-specific-IgG, -IgG_1, -IgG_2, -IgG_3 and -IgG_4 among subjects with different biting reactions. Midge extract elicited significantly more PBMC proliferation, higher expression of IFN-γ, IL-10, IL-6 and TNF-α in SDR subjects than in NAC. Protease activity was detected in midge extract. Protease inhibitors E64 and pepstatin suppressed midge-extract-induced IL-8 production. ***Conclusions:*** Our results suggest that an immediate reaction to midge bites is IgE-mediated. IFN-γ, IL-6 and TNF-α are involved in delayed reactions to midge bites. A protease-activated pathway may also be involved in the intense, itchy reactions to midge bites.

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Introduction

Forcipomyia taiwana is a tiny (1–1.5 mm) blood-sucking midge which is widely found in urban and suburban habitats in Taiwan and southern China [1, 2]. Like mosquito bites, midge bites can cause allergic reactions. During the day, groups of *F. taiwana* attack exposed parts of the human body [3], causing intense pruritis and swelling in allergic individuals.

There are 2 types of reactions after midge bites: (1) immediate reaction, in which a large local swelling develops within 1 h of bites, and (2) delayed reaction, in which intense itching papules and vesicles develop 6–24 h after

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bites. The delayed-type lesions may turn centrally necrotic several days later and last for weeks or even months. We have previously reported that about 59.1% of exposed subjects develop hypersensitivity reactions to midge bites [4]. Among the midge-allergic subjects, 14% developed a solely immediate reaction, 43% developed an immediate reaction followed by delayed reactions and 43% developed a solely delayed reaction. Subjects with insect allergy frequently curtail outdoor activities because of the annoying allergic reactions, leading to a significant worsening of their quality of life [5].

Our previous study identified the 24-, 35/36- and 64-kDa proteins as major IgE-binding allergens of this midge [4]. However, the immune mechanism of delayed reactions to midge bites has not been well studied. Delayed reactions to midge bites usually last for weeks or even months, causing an even greater impact on the patient's quality of life than the rapidly resolved immediate reaction. Except for a few studies that focused on the immune mechanisms of mosquito bites [6–12], to the best of our knowledge, there have been no reports in the medical literature regarding the immune responses to midge bites in humans. In the present study, we examined specific IgE and IgG responses in subjects with allergic reactions to *F. taiwana* and cytokine changes in subjects with delayed reactions in order to further understand the immune mechanisms involved.

Materials and Methods

Preparation of Whole Body Midge Extract

Female *F. taiwana* were collected by the human bait method using special collectors designed by Dr. C.S. Chen's laboratory, Department of Life Science, Tunghai University, Taichung, Taiwan. One thousand female midges were ground and suspended in 5 ml of PBS, ultrasonicated for 30 min at 4°C and centrifuged at 8,000 *g* for 15 min. The supernatant was collected, filtered through a 0.22-μm filter, aliquoted and stored at –70°C. The protein concentration was determined with a Bradford assay reagent (Bio-Rad, Hercules, Calif., USA) [13].

Subjects

The project was reviewed and approved by the institutional review board of Taichung Veterans General Hospital. Recruitment of volunteer human subjects who had been exposed to *F. taiwana* bites was done through 2 channels: advertisement and the allergy clinic of our hospital. They were interviewed by a nurse regarding details of their reactions to midge bites and the skin lesions caused by midge bites in natural settings were examined by a physician (Y.-H.C.). Subjects were then skin-tested with the midge extract after giving their written informed consent. For the skin test we used midge extract at a concentration of 200 μg/ml in PBS containing 50% glycerol, with epicutaneous sterile disposable Sharp Test® applicators (Greer Laboratories, Lenoir, N.C., USA). Histamine (1 mg/ml) and 50% PBS-glycerol were used as positive and negative controls, respectively. All skin test results were read 20 min after placement for immediate wheal-and-flare reaction. A response with a wheal or an erythema 3 mm larger in size than that produced by the negative control was considered positive. 30–50 ml of blood was drawn after the skin test.

Subjects were divided into 3 groups based on their clinical symptoms and skin lesions: (1) those with an immediate reaction, regardless of the presence of delayed reactions; (2) those with solely delayed reactions, and (3) those who tolerated and had no reaction to *F. taiwana* bites. They constituted the nonallergic control group.

Detection of Midge-Specific IgE, IgG and IgG Subclasses

Indirect ELISA was used to determine the *F. taiwana* (midge)-specific IgE, IgG and IgG subclasses. After choosing optimal conditions by using checkerboard titration, microtiter plates (Nunc, Roskilde, Denmark) were coated with 3 μg/well midge extract in triplicate and incubated at 37°C for 2 h. In general, 3 washings were performed between each step. After blocking with 1% goat serum, the plates were incubated with 1:10 dilution of sera from subjects and incubated for 2 h at room temperature. After washing, antibody binding was detected using alkaline phosphatase-labeled mouse anti-human IgE (BD Pharmingen, San Diego, Calif., USA), IgG, IgG_1, IgG_2, IgG_3 or IgG_4 antibodies (Zymed Laboratories, San Francisco, Calif., USA) respectively, along with ρ-nitrophenyl phosphate (Sigma, St. Louis, Mo., USA) as substrate. The optical density (OD) was determined at 405 nm on a microtiter plate reader (Tecan, Grödig, Austria).

Preparation of PBMC

Peripheral blood mononuclear cells (PBMC) from subjects were separated from heparinized blood by centrifugation through Ficoll-Paque solution (Pharmacia, Uppsala, Sweden). After 3 washings with PBS, the cells were suspended in conditioned media composed of RPMI (Sigma) supplemented with 2 g/ml sodium bicarbonate, 2 mM L-glutamine, 5 mM HEPES buffer, 200 U/ml penicillin, 200 μg/ml streptomycin and 10% heat-inactivated autologous human serum.

Cell Culture and PBMC Proliferation by MTT Assay

PBMC (2×10^5) were cultured in 96-well flat-bottomed culture plates in duplicate at 37°C in an incubator with 5% CO_2 with: (1) media alone; (2) 0.2 μg/ml concanavalin A; (3) 0.3 μg/ml midge extract; (4) 3 μg/ml midge extract, or (5) 30 μg/ml midge extract. After 24 and 48 h of culture, 20 μl (5 mg/ml) of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; Sigma) was added into each well and incubated for an additional 1 h. 100 μl of dimethyl sulfoxide was added to dissolve the formazan crystals in viable cells. The absorbance of the converted dye was read at a wavelength of 570 nm with background subtraction at 650 nm with a microtiter plate reader (Tecan). Data were presented as a stimulation index by the following calculation: level of $OD_{570\,nm}$ in medium with midge extract/level of $OD_{570\,nm}$ in medium only at same time point [14].

Cell Culture for Cytokine Analysis

PBMC (1×10^6) were cultured in 12 × 75 mm BD Falcon™ cell culture tubes (BD Biosciences, Franklin Lakes, N.J., USA) in

duplicate in 1 ml of media alone, with concanavalin A (0.2 μg/ml) or midge extract (3 μg/ml) for 24, 72 and 144 h. Phorbol 12-myristate-13-acetate (PMA, 50 ng/ml) was then added and incubated for an additional 24 h. The samples were then pooled from the duplicate cultures and harvested. The cells were centrifuged and preserved in RNAlater (Ambion, Foster City, Calif., USA) for RNA extraction. The cell-free supernatants were stored at –70°C until further analysis.

RNA Extraction and cDNA Synthesis

Total RNA from PBMC was extracted using RNAzol reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, Calif., USA). The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Beckman DU7400). cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction mixture containing 2.5 μM of oligo-dT primer, 1 mM deoxyribonucleoside triphosphates (dNTP), 5 μM dithiothreitol, 40 U of RNaseOUT RNase inhibitor, and 15 U of ThermoScript reverse transcriptase (Invitrogen). Reverse transcription was performed at 50°C for 60 min followed by a heat denaturation step at 85°C for 5 min with a thermocycler (Perkin-Elmer, Irvine, Calif., USA).

Cytokine mRNA Expression by Real-Time PCR

mRNA levels were quantified by subjecting cDNA to TaqMan PCR analysis using a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Überlingen, Germany). Predesigned sequence detection reagents specific for human IFN-γ, IL-10, IL-6 and TNF-α were purchased from Applied Biosystems. To normalize each sample for RNA content, we used β-actin as a control gene. Each probe has a fluorescent reporter dye (FAM) linked to its 5′ end and a downstream quencher dye (TAMRA) linked to its 3′ end. Each 20-μl PCR reaction mix included 1 × TaqMan Universal Master Mix with AmpliTaq Gold DNA polymerase, 1 × probe/primer mix and 50 ng cDNA. Amplification conditions consisted of 45 cycles of 95°C for 15 s and 60°C for 1 min after incubation at 95°C for 10 min. The threshold cycle C(t), the cycle number at which there was a detectable increase above background fluorescence, was determined for each sample using GeneAmp software. The results were expressed as fold changes between medium control and midge extract-stimulated cells after correcting by the housekeeping gene.

Cytokine Proteins in Culture Supernatants Using Cytometric Bead Immunoassay

Culture supernatant was analyzed simultaneously for 11 different cytokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IFN-γ, TNF-α and TNF-β, using the bead-based FlowCytomix (Bender MedSystems, Vienna, Austria) as described previously [15, 16]. Following a final wash, the contents of each well were suspended in 200 μl of assay buffer, placed in 12 × 75-mm polystyrene tubes, and then read on a FACScan™ flow cytometer (BD Biosciences). Cytokine concentrations were calculated by comparison with a standard curve for each cytokine derived from the cytokine standards assayed in the same manner.

Detection of Protease Activity in Midge Extract by Azocoll Method

Protease activity was determined by the Azocoll (Sigma) method as described previously [17]. Protease activity from partially purified cockroach allergen Cr PI [18, 19] was measured concomitantly for comparison. A different concentration of subtilisin (Sigma) from *Bacillus subtilis* was used as a standard.

Effects of Protease Inhibitors on Midge Extract-Induced IL-8 Release by A549 Cells

The A549 airway-derived epithelial cell line, purchased from the Bioresource Collection and Research Center, Taiwan, was grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. For the experiments, the cells were seeded in 6-well culture plates and grown to 80% confluence. To test the effect of protease inhibitors, midge extract was pretreated for 15 min at 37°C with or without the inhibitors before being added to A549 cells for 24 h. Culture supernatants were collected and stored at –70°C until assayed. The following protease inhibitors were used: phenylmethane sulfonyl fluoride (PMSF; 0.01–0.4 nM), E-64 (5–200 ng) and pepstatin (5–200 ng). All protease inhibitors were obtained from Sigma and dissolved in ion-free PBS (Dulbecco's PBS; Gibco, Invitrogen) before use. The concentration of IL-8 in the culture supernatants were measured using an ELISA kit (Bender) according to the manufacturer's instructions.

Statistical Analysis

Data in the figures are presented as mean ± standard error of mean (SEM) unless otherwise specified. Medians and ranges were described in the text. Differences of means were compared using a nonparametric Mann-Whitney U test. Analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, Ill., USA). A p value less than 0.05 was considered statistically significant.

Results

Clinical Data and Midge-Specific IgE and IgG Levels

Sixty-two subjects were enrolled and subgrouped according to their reactions to midge bites. As shown in table 1, 20 subjects had an immediate reaction with or without a delayed reaction, 12 subjects had solely delayed reactions (SDR) and 30 subjects had a tolerance to midge bites, and so were nonallergic controls (NAC). Sera were collected and tested for midge-specific IgE, IgG and IgG subclasses. The mean level of midge-specific IgE was significantly elevated among subjects with an immediate reaction to midge bites, regardless of whether they had subsequent delayed reactions or not (OD = 0.37 ± 0.06, median 0.31, range 0.1–1.08). Comparably low levels of midge-specific IgE were found in SDR (OD 0.10 ± 0.01, median 0.10, range 0.05–0.16) and NAC subjects (OD 0.07 ± 0.01, median 0.06, range 0.05–0.09; fig. 1a). There were no differences with regard to the level of midge-specific IgG, IgG_1, IgG_2, IgG_3 and IgG_4 among the 3 groups (fig. 1b).

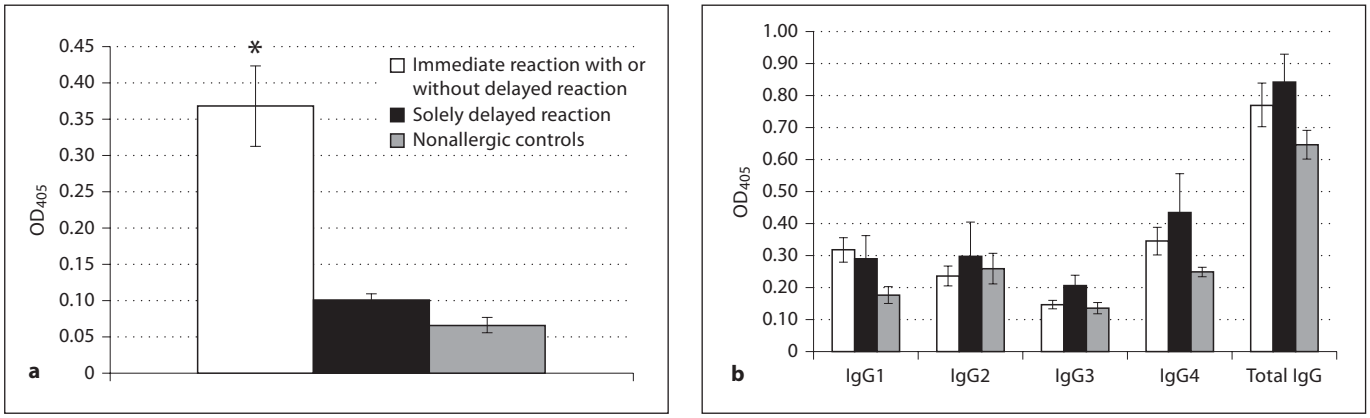


Fig. 1. Midge-specific IgE (**a**) and IgG subclasses (**b**) in individuals with different allergic reactions to midge bites and nonallergic subjects by ELISA. Results are expressed as mean ± SEM from 20 midge-allergic subjects with immediate reaction, with or without delayed reaction, 12 subjects with solely delayed reaction and 30 nonallergic subjects. * $p < 0.05$ versus nonallergic subjects.

Table 1. Patient demographics

Reaction after midge bite	Immediate allergic reaction ± delayed reaction (n = 20)	Solely delayed reaction (n = 12)	Nonallergic control (n = 30)
Age (mean ± SD), years	32.1 ± 2.47	37.75 ± 2.54	29.1 ± 2.01
Gender, n			
Male	4	2	15
Female	16	10	15
SPT to crude midge extract			
Wheal size (mean ± SD), mm	6.24 ± 0.38	0.25 ± 0.18	0 ± 0
Erythema (size ± SD), mm	15.82 ± 2.12	0.33 ± 0.22	0.09 ± 0.06

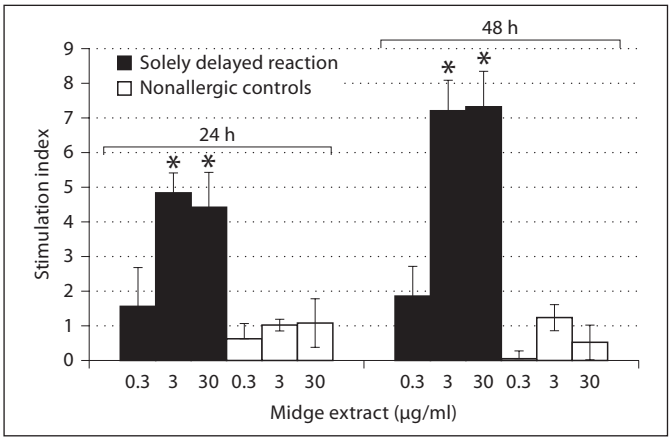


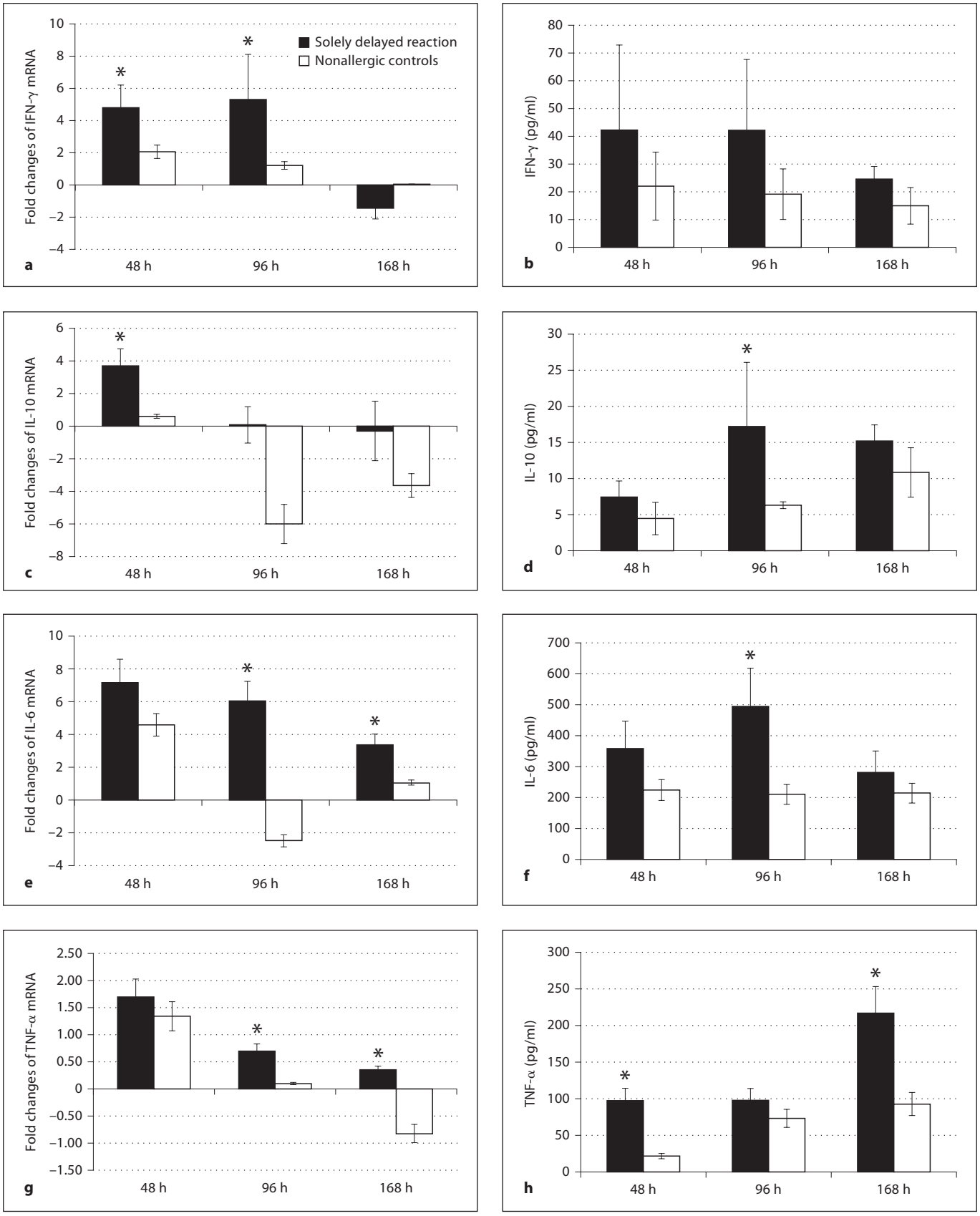
Fig. 2. PBMC proliferation from 6 midge-allergic subjects with solely delayed reaction and 6 nonallergic subjects after stimulation with various concentrations of midge extract. * $p < 0.05$ versus nonallergic subjects.

Proliferation of PBMC

PBMC from 6 SDR subjects and 6 NAC subjects were stimulated with different concentrations of midge extract and then cell proliferation was determined by MTT assay. We found that PBMC from SDR subjects markedly proliferated in response to midge extract compared to NAC at 3 μg/ml at 24 h [stimulation index 4.83 ± 2.44 vs. 1.02 ± 0.16, median (range) 4.43 (0.42–18.13) vs. 0.85 (0.33–1.35)] and 48 h [stimulation index 7.22 ± 2.78 vs. 1.24 ± 0.38, median (range) 5.09 (1.94–22.48) vs. 0.98 (0.49–2.00)]. A higher concentration of 30 μg/ml gave no further increase of cell proliferation, as shown in figure 2.

Cytokine Profile of SDR Subjects

PBMC from 6 SDR subjects and 6 NAC subjects were stimulated with midge extract for 48, 96 and 168 h. As shown in figure 3a, c, e and g, mRNA from SDR subjects


Solely delayed reaction
Nonallergic controls
Fold changes of IFN-γ mRNA
IFN-γ (pg/ml)
Fold changes of IL-10 mRNA
IL-10 (pg/ml)
Fold changes of IL-6 mRNA
IL-6 (pg/ml)
Fold changes of TNF-α mRNA
TNF-α (pg/ml)
48 h
96 h
168 h
a
b
c
d
e
f
g
h

3

expressed more IFN- γ, IL-10, IL-6 and TNF-α than NAC subjects at 48, 96 and 168 h. The median fold changes (range) are as follows (SDR vs. NAC).

IFN-γ at 48 h: 4.66 (1.22 to 9.99) versus 2.06 (–1.03 to 4.76), at 96 h: 4.89 (–1.44 to 16.68) versus 1.38 (–7.06 to 5.03), and at 168 h: –1.36 (–4.06 to 1.21) versus –1.03 (–3.94 to 7.57).

IL-10 at 48 h: 3.22 (1.33 to 7.62) versus 0.08 (–2.19 to 1.36), at 96 h: –0.57 (–2.20 to 4.32) versus –2.26 (–35.26 to 10.20), and at 168 h: 0.02 (–5.37 to 6.19) versus –3.65 (–15.78 to 6.41).

IL-6 at 48 h: 7.63 (–5.98 to 19.43) versus 2.68 (–1.45 to 12.55), at 96 h: 2.20 (–2.60 to 29.65) versus –1.69 (–19.4 to 10.03), and at 168 h: 1.85 (–1.59 to 13.74) versus 1.05 (–1.43 to 6.36).

TNF-α at 48 h: 1.61 (1.01 to 2.71) versus 1.34 (1.04 to 1.88), at 96 h: 0.87 (–1.93 to 2.95) versus –0.30 (–4.26 to 1.82), and at 168 h: 1.2 (–0.35 to 2.38) versus –0.90 (–4.20 to 2.08).

As shown in figure 3b, d, f and h, PBMC from SDR subjects secreted more IFN-γ, IL-10, IL-6 and TNF-α than NAC subjects at 48, 96 and 168 h. The differences reached statistical significance at 96 h in IL-10 and IL-6 and at 48 h and 168 h in TNF-α. The mean fold changes (range) were as follows (SDR versus NAC).

IFN-γ at 48 h: 51 pg/ml (24–109) versus 22 pg/ml (5–43), at 96 h: 43 pg/ml (29–94) versus 21 pg/ml (5–31), and at 168 h: 26 pg/ml (25–64) versus 16 pg/ml (5–24).

IL-10 at 48 h: 8 pg/ml (5–10) versus 7 pg/ml (2–10), at 96 h: 19 pg/ml (16–39) versus 7 pg/ml (6–8), and at 168 h: 16 pg/ml (8–23) versus 14 pg/ml (4–25).

IL-6 at 48 h: 298 pg/ml (104–835) versus 210 pg/ml (35–346), at 96 h: 340 pg/ml (190–866) versus 215 pg/ml (80–222), and at 168 h: 244 pg/ml (51–698) versus 208 pg/ml (16–298).

TNF-α at 48 h: 91 pg/ml (63–263) versus 16 pg/ml (8–42), at 96 h: 89 pg/ml (67–257) versus 68 pg/ml (10–196), and at 168 h: 213 pg/ml (102–635) versus 87 pg/ml (9–152).

Fig. 3. Cytokine mRNA and protein expression from PBMC of 6 SDR and 6 NAC subjects. PBMC were cultured with midge extract (3 μg/ml) for various time points and mRNA expression was quantified by real-time PCR and assayed cytokine proteins by cytometric bead immunoassay. **a** IFN-γ mRNA. **b** IFN-γ protein. **c** IL-10 mRNA. **d** IL-10 protein. **e** IL-6 mRNA. **f** IL-6 protein. **g** TNF-α mRNA. **h** TNF-α protein. Data are mean ± SEM. * $p < 0.05$ versus nonallergic subjects.

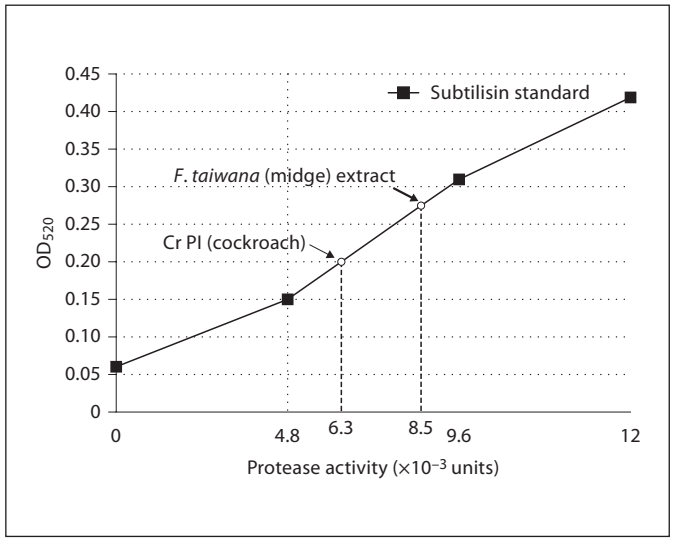


Fig. 4. Protease activity in midge extract (20 μg/ml) by the Azocoll method. Thick and thin arrows indicate the estimated protease activity of midge extract (8.5×10^{-3} units) and Cr PI (cockroach allergen, 6.3×10^{-3} units), respectively.

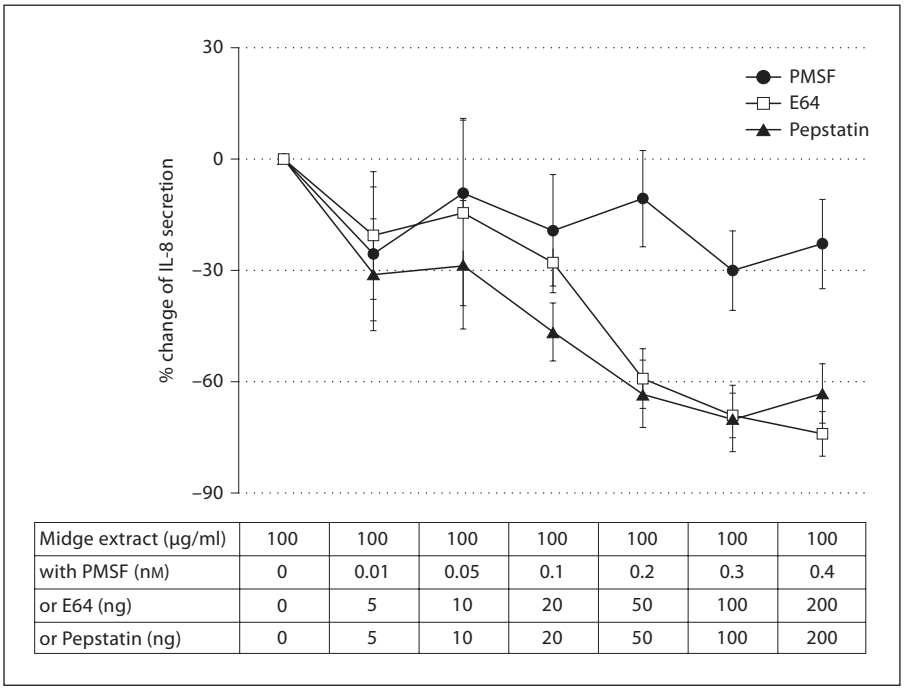
IL-8 (13,102.38 pg/ml vs. 11,200.43 pg/ml at 48 h) and IL-2 (60.27 pg/ml vs. 70.52 pg/ml at 48 h) increased in both SDR and NAC subjects, with no significant differences. IL-4, IL-5, IL-12p70 and TNF-β were not detected in the culture supernatant (data not shown).

Protease Activity in Midge Extract

Whole body midge extract stimulated IL-8 production at high levels from PBMC in both the midge-allergic and nonallergic subjects. A similar pattern has been reported in cockroach allergens that contain protease activity [20, 21], thus we decided to determine the protease activity in midge extract and used cockroach allergen Cr PI for comparison. Protease activity was detected in midge extract by the Azocoll method. The protease activity was higher in the midge extract (8.5×10^{-3} units) than in cockroach allergen Cr PI (6.3×10^{-3} units; fig. 4).

Inhibition of Midge-Extract Induced IL-8 Secretion by Protease Inhibitors from A549 Cells

IL-8 production from A549 cells stimulated by midge extract was suppressed by E-64 and pepstatin in a dose-dependent manner but not PMSF, as shown in figure 5.



Midge extract (μg/ml)	100	100	100	100	100	100	100
with PMSF (nM)	0	0.01	0.05	0.1	0.2	0.3	0.4
or E64 (ng)	0	5	10	20	50	100	200
or Pepstatin (ng)	0	5	10	20	50	100	200

Fig. 5. Effects of protease inhibitors on midge extract-induced IL-8 release from A549 cells. Midge extract (100 μg/ml) was incubated for 15 min at 37°C with different kinds and different amounts of protease inhibitors, PMSF (serine and cysteine protease inhibitor), E64 (papain and cysteine protease inhibitor) or pepstatin (aspartic protease inhibitor), as indicated in the table below the figure, then cultured with A549 cells for additional 24 h. Data are mean ± SEM, n = 3.

Discussion

Allergic reactions to midge bites are a common problem in Taiwan. In this study, we found that the mean midge-specific-IgE was significantly higher in subjects with an immediate reaction, which is consistent with the previous findings of mosquito allergy [22]. In our study, midge-specific IgG and midge-specific IgG_4 were elevated in all 3 groups regardless of their reactions to midge bites. It is possible that the presence of midge-specific IgG and midge-specific IgG_4 only correlates to exposure to midge bites, but not to the presence of allergic symptoms.

It has been demonstrated in a mouse model of mosquito allergy that IL-4 production significantly increased and IFN-γ production decreased [23]. This study suggested a predominant Th2 immune response in mice with delayed skin reactions to mosquito bites. However, in our ex vivo experimental model, midge extract stimulated more IFN-γ, IL-10, IL-6 and TNF-α from PBMC in human subjects with SDR than nonallergic subjects at both mRNA and protein levels. IFN-γ is considered a cytokine indicating Th1 type inflammation and TNF-α and IL-6 are proinflammatory cytokines mainly secreted by innate immune cells [24, 25]. Nevertheless, since we did not study the cellular responses of subjects with solely immediate reactions, we cannot exclude the possibility that IL-4 or other Th2 cytokines participate in the immediate reaction of biting midge allergy.

TNF-α is a key molecule in many inflammatory diseases. The evidence of TNF-α in allergic inflammation has been increasing, especially in refractory asthma [26]. However, the study regarding TNF-α in biting or stinging-insect allergy is limited. Only one previous report has described that human T cell clones specific to bee venom phospholipase A2 produced TNF-α in response to allergen [27]. It has been demonstrated that mast-cell derived TNF-α induced eosinophil survival by autocrine production of granulocyte-macrophage colony-stimulating factor [28]. Our study suggested that TNF-α may play a role in the dense eosinophil infiltration in the skin lesions of subjects with a delayed allergic reaction to biting midge.

IL-10 has been shown to be an anti-inflammatory cytokine produced by regulatory T cells. It inhibits the activation and cytokine production of both Th1 and Th2 cells [29, 30]. It is also considered to play an important role in immune tolerance in allergic inflammation and allergen-specific immunotherapy [31]. However, IL-10 does not seem to be a good indicator for the existence of immune tolerance to midge bites in our study. SDR subjects in our study secreted more IL-10 than the midge-tolerant nonallergic subjects. An increasing secretion of

IL-10 seems to be more of a sign that the immune system 'sees' the allergen and tries to stop the inflammation rather than a successful induction of tolerance to the midge allergy.

In the present study, protease activity was detected in midge extract, and the midge extract-induced IL-8 secretion was suppressed by cysteine and aspartic protease inhibitor. It is known that extracellular endogenous proteases, such as thrombin and trypsin, as well as exogenous proteases from cockroaches [20, 32], mites [33] and mold allergens [34], react with cell surface receptors in the airways and generate leukocyte infiltration and amplify the response to allergens. There has been increasing evidence that protease activation inflammation plays an important role in non-IgE mediated allergic respiratory diseases via activation of protease-activated receptors [35]. The activation of protease activated receptor-2 in skin has been reported to be related to skin itching, recruitment of inflammatory cells and hyper-pigmentation [36–38]. With the detection of strong protease activity in crude *F. taiwana* extracts, we believe that protease activation of inflammation plays an important role in the delayed, long-lasting, intensely itchy, hyperpigmented allergic skin reactions after *F. taiwana* bites. Since protease activation of inflammation is non-specific, it is possible that there is a difference, as yet to be defined, in negative regulatory signals in NAC subjects that mediate the tolerance to midge bites.

In conclusion, our results suggest that an immediate reaction to midge bites is IgE-mediated, whereas IgE may not be the major molecule in delayed reactions. IFN-γ, IL-6, and TNF-α are involved in delayed reactions to midge bites. A protease-activated pathway may also be involved in the intense, itchy reactions to midge bites.

Acknowledgments

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References

1 Chuang YY, Lin CS, Wang CH, Yeh CC: Distribution and seasonal occurrence of *Forcipomyia taiwana* (Diptera: Ceratopogonidae) in the Nantou area in Taiwan. J Med Entomol 2000;37:205–209.
2 Yeh CC, Chuang YY: Colonization and bionomics of *Forcipomyia taiwana* (Diptera: Ceratopogonidae) in the laboratory. J Med Entomol 1996;33:445–448.
3 Chen CS, Lien JC, Lin YN, Hsu SJ: The diurnal biting pattern of a bloodsucking midge *Forcipomyia (Lasiohelea) taiwana (Shiraki)* (Diptera, Ceratopogonidae). Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 1981;14:54–56.
4 Chen YH, Lee MF, Lan JL, Chen CS, Wang HL, Hwang GY, Wu CH: Hypersensitivity to *Forcipomyia taiwana* (biting midge): clinical analysis and identification of major For t 1, For t 2 and For t 3 allergens. Allergy 2005;60:1518–1523.
5 Oude Elberink JN, Dubois AE: Quality of life in insect venom allergic patients. Curr Opin Allergy Clin Immunol 2003;3:287–293.
6 Suzuki S, Negishi K, Tomizawa S, Shibasaki M, Kuroume T: A case of mosquito allergy. Immunological studies. Acta Allergol 1976;31:428–441.
7 Peng Z, Yang M, Simons FE: Measurement of mosquito *Aedes vexans* salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. Ann Allergy Asthma Immunol 1995;74:259–264.
8 Peng Z, Yang M, Simons FE: Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens. Ann Allergy Asthma Immunol 1996;77:238–244.
9 Kuraishi Y, Ohtsuka E, Nakano T, Kawai S, Andoh T, Nojima H, Kamimura K: Possible involvement of 5-lipoxygenase metabolite in itch-associated response of mosquito allergy in mice. J Pharmacol Sci 2007;105:41–47.
10 Peng Z, Simons FE: Advances in mosquito allergy. Curr Opin Allergy Clin Immunol 2007;7:350–354.
11 Peng Z, Simons FE: Mosquito allergy: immune mechanisms and recombinant salivary allergens. Int Arch Allergy Immunol 2004;133:198–209.
12 Palosuo K, Brummer-Korvenkontio H, Mikkola J, Sahi T, Reunala T: Seasonal increase in human IgE and IgG4 antisaliva antibodies to *Aedes* mosquito bites. Int Arch Allergy Immunol 1997;114:367–372.
13 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
14 Hay FC, Westwood OMR (eds): Practical Immunology. Oxford, Blackwell Science, 2002, pp 261–300.
15 Kellar KL, Kalwar RR, Dubois KA, Crouse D, Chafin WD, Kane BE: Multiplexed fluorescent bead-based immunoassays for quantitation of human cytokines in serum and culture supernatants. Cytometry 2001;45:27–36.
16 Yang DL, Kraght P, Pentoney CS, Pentoney SL Jr: Analytical significance of encroachment in multiplexed bead-based flow cytometric assays. Anal Chem 2007;79:3607–3614.
17 Chavira R Jr, Burnett TJ, Hageman JH: Assaying proteinases with azocoll. Anal Biochem 1984;136:446–450.
18 Wu CH, Lan JL: Cockroach hypersensitivity: isolation and partial characterization of major allergens. J Allergy Clin Immunol 1988;82:727–735.

19 Jeng KC, Liu MT, Wu CH, Wong DW, Lan JL: American cockroach Cr-PI allergen induces lymphocyte proliferation and cytokine production in atopic patients. Clin Exp Allergy 1996;26:349–356.

20 Page K, Hughes VS, Odoms KK, Dunsmore KE, Hershenson MB: German cockroach proteases regulate interleukin-8 expression via nuclear factor for interleukin-6 in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2005;32:225–231.

21 Page K, Strunk VS, Hershenson MB: Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. J Allergy Clin Immunol 2003;112:1112–1118.

22 Peng Z, Beckett AN, Engler RJ, Hoffman DR, Ott NL, Simons FE: Immune responses to mosquito saliva in 14 individuals with acute systemic allergic reactions to mosquito bites. J Allergy Clin Immunol 2004;114:1189–1194.

23 Chen YL, Simons FE, Peng Z: A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. Int Arch Allergy Immunol 1998;116:269–277.

24 Locksley RM, Killeen N, Lenardo MJ: The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001;104:487–501.

25 Doganci A, Sauer K, Karwot R, Finotto S: Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. Clin Rev Allergy Immunol 2005;28:257–270.

26 Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ, Pavord ID: Evidence of a role of tumor necrosis factor alpha in refractory asthma. N Engl J Med 2006;354:697–708.

27 Carballido JM, Carballido-Perrig N, Terres G, Heusser CH, Blaser K: Bee venom phospholipase A2-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to the antigen concentration. Eur J Immunol 1992;22:1357–1363.

28 Levi-Schaffer F, Temkin V, Malamud V, Feld S, Zilberman Y: Mast cells enhance eosinophil survival in vitro: role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. J Immunol 1998;160:5554–5562.

29 Akdis CA, Joss A, Akdis M, Blaser K: Mechanism of IL-10-induced T cell inactivation in allergic inflammation and normal response to allergens. Int Arch Allergy Immunol 2001;124:180–182.

30 Jutel M, Akdis M, Budak F, Ebischer-Casaulta C, Wrzyszcz M, Blaser K, Akdis CA: IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205–1214.

31 Akdis CA, Blaser K: IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. FASEB J 1999;13:603–609.

32 Bhat RK, Page K, Tan A, Hershenson MB: German cockroach extract increases bronchial epithelial cell interleukin-8 expression. Clin Exp Allergy 2003;33:35–42.

33 Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, Stewart GA: House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. J Immunol 2002;169:4572–4578.

34 Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P: Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. J Allergy Clin Immunol 2000;105:1185–1193.

35 Reed CE, Kita H: The role of protease activation of inflammation in allergic respiratory diseases. J Allergy Clin Immunol 2004;114:997–1008.

36 Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, Luger TA, Schmelz M: Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 2003;23:6176–6180.

37 Babiarz-Magee L, Chen N, Seiberg M, Lin CB: The expression and activation of protease-activated receptor-2 correlate with skin color. Pigment Cell Res 2004;17:241–251.

38 Steinhoff M, Corvera CU, Thoma MS, Kong W, McAlpine BE, Caughey GH, Ansel JC, Bunnett NW: Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. Exp Dermatol 1999;8:282–294.

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學歷

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5. 中華民國醫學教育學會

6. 中華民國超音波醫學會

研究計畫

1. 國科會計畫 2006-2009 (三年), 計畫主持人 NSC-95-2314-B-075A-016-MY3, 以 DNA 重組技術建立台灣鈸蠓(小黑蚊)過敏之標準檢驗方法
2. 國科會計畫 2008-2011 (三年), 共同主持人,NSC-97-2320-B-075A-001-MY3 蟑螂專一性細胞株及蟑螂過敏疫苗之研究,
3. 台中榮總院內計畫 2008, 計畫主持人 TCVGH-97302C, 蛋白胺活化發炎反應在台灣鈸蠓(小黑蚊)過敏免疫機轉之研究
4. 國科會計畫 2007, 共同主持人,以 Pera 1, Pera 3 及 Pera 7 重組過敏原診斷蟑螂過敏及致敏機轉探討
5. 台中榮總院內計畫 2007, 計畫主持人 TCVGH-96302C, 台灣鈸蠓(小黑蚊)過敏免疫機轉之研究
6. 台中榮總院內計畫 2006, 計畫主持人 TCVGH-95304C, 台灣鈸蠓(小黑蚊)過敏原 Fort2 之分子選殖
7. 國科會計畫 2004-2005 (兩年), 計畫主持人 NSC-94-2314-B-075A-001, 台灣鈸蠓(小黑蚊)過敏原之鑑定、選殖與臨床特徵
8. 台中榮總院內計畫 2005, 計畫主持人，TCVGH-943804B：以胜肽指紋分析法鑑定台灣鈸蠓(小黑蚊)過敏原
9. 衛生署國民健康局 2004, 計畫主持人：醫療從業人員乳膠過敏之篩檢
10. 國科會計畫, 2002, 協同主持人 NSC 91-2320-B-075A-002: 乳膠印度棗交叉過敏原之鑑定與選殖
11. 台中榮總院內計畫 2002, 計畫主持人 TCVGH-91913802A: 台灣乳膠水果症候群盛行率之研究
12. 台中榮總院內計畫 2001, 計畫主持人 TCVGH-903802B：過敏病患與健康捐血者乳膠過敏流行病學之研究
13. 台中榮總院內計畫 1999, 計畫主持人 TCVGH-883803: 抗中性球細胞質抗體在全身性紅斑狼瘡之研究

獲獎:

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Publications

期刊論文

1. **Y.H. Chen**, M.F. Lee, J.J. Tsai, H.J. Wu,G.Y. Hwang. Specific IgE and IgG responses and cytokine profile in subjects with allergic reactions to biting midge *Forcipomyia taiwana*. *Int Arch Allergy Immunol.* ; 150(1):66-74. 2009 (SCI)
2. **Y.H. Chen**, M.F. Lee, H. J. Wu, J.J. Tsai. Anaphylactic shock to a 33-kDa alpha-S1-casein-like protein in kingfish caviar. *Journal of Investigational Allergology and Clinical Immunology*, 9 (3), May-June 2009 (SCI)
3. T.M. Yu, **Y.H. Chen**, J.Y. Hsu, Y.W. Chuang, C.H. Cheng, C.H. Chen and K.H. Shu Systemic Inflammation is Associated with Pulmonary Hypertension in Patients Undergoing Hemodialysis. *Nephrol Dial Transplant* .Accepted Dec. 2008.(SCI)
4. C. T. Lin, D. Z. Hung, D. Y. Chen, H. J. Wu, J. L. Lan, and **Y. H. Chen***. A hospital-based screening study of latex allergy and latex sensitization among

medical workers in Taiwan. *J Microbiol.Immunol Infect.* 41 (6):499-506, 2008.

5. M.F. Lee, J.J. Tsai, G.Y. Hwang, S.J. Lin, **Y.H. Chen***.Identification of immunoglobulin E (IgE)-binding epitopes and recombinant IgE reactivities of a latex cross-reacting Indian jujube Ziz m 1 allergen. *Clin Exp Immunol.* 2008;152(3):464-71 (SCI).
6. T.M. Yu, S.H. Lin, Y.W. Chuang, M.C.Wen, **Y.H. Chen**, C.H. Cheng,C.H. Chen, C.S. Chin and K.H. Shu. A syndrome resembling Bartter's syndrome in sarcoidosis. *Nephrol Dial Transplant* .2008. Nov 5. [Epub ahead of print] (SCI)
7. T.M. Yu, **Y.H. Chen**, J.L.Lan, C.H. Cheng, C.H. Chen, M.J. Wu, K.H. Shu. Renal outcome and evolution of disease activity in Chinese lupus patients after renal transplantation. *Lupus.* ;2008；17(7):687-94.(SCI)
8. T.W. Hseun, D.Y. Chen, T.Y. Hsieh, W.N. Huang, **Y.H. Chen***, J.L. Lan. Hypereosinophilic syndrome in a medical center in Taiwan—an analysis of fourteen cases. *Formosan Journal of Rheumatology* 2008;22:60-67
9. H.K.Lin, J.L.Lan, D.Y. **Chen, Y.H.** Chen, W.N. Huang, T.Y. Hsieh, C.W. Hsieh, H.H. Chen. The diagnostic value of anti-cyclic citrullinated peptide antibodies and rheumatoid factor in patients with rheumatoid arthritis. *Formosan Journal of Rheumatology* 2008;22:68-73
10. 藍忠亮、陳得源、陳怡行.新進醫事人員訓練現況與發展—醫院臨床訓練教育的轉變。醫療品質雜誌 2008; 2(6):13-16
11. 翁嘉澤 **陳怡行** 劉明輝. 慢性特發性蕁麻疹. 內科學誌. 2008 年 4 月
12. C.H.Chen, **Y.H. Chen**, J.L.Lan, D.Y. Chen*. Scleroderma renal crisis: a rare complication of systemic sclerosis with poor prognosis-experience in a medical center in central Taiwan. *Journal of Rheumatology. R.O.C.* 2007;21:59-64.
13. C.T. Lin, **Y.H. Chen***, D.Y. Chen, W.N. Huang, J.L.Lan. Low dose intravenous

cyclophosphamide-induced ovarian failure in Chinese patients with lupus nephritis. *Journal of Rheumatology. R.O.C*. 2007;21:53-58.

14. C.H.Tung, **Y.H,Chen**, H.H.C. Lan, T.Y. Hsieh, D.Y. Chen, J.L. Lan. Diagnosis of plant thorn synovitis by high-resolution ultrasonography – a case report and literature review *Clin Rheumatol*. 2007,26:849-851.（SCI）
15. K.D. Hong, **Y.H. Chen**, D.Y. Chen, J.L. Lan. Subcutaneous panniculitis-like T-cell lymphoma presenting with hemophagocytic lymphohistiocytic and skin lesions with characteristic high-resolution ultrasonographic findings. *Clin Rheumatol.* 2007,26:775-778（SCI）
16. M.F. Lee, **Y. H. Chen**, H.C. Lin, H.L. Wang, G.Y. Hwang, C.H. Wu. Identification of Hevamine and Hev b1 as major latex allertens in Taiwan. *Int Arch Allergy Immunol* 2006;139(1):38-44. (SCI)
17. M.F. Lee, G.Y. Hwang, **Y.H.Chen,** H.C. Lin, C.H. Wu. Molecular cloning of Indian jujube(Zizyphus mauritiana) Ziz m 1 with sequence similarity to plant class III chitinase. *Molecular Immunology*. 2006, Mar; 43(8):1144-51 (SCI)
18. **Y. H. Chen**, M. F. Lee, J. L. Lan, C.S.Chen, H.L. Lin, G. Y. Huang, and C. H. Wu. Hypersensitivity to Forcipomyia taiwana (biting midge): Clinical analysis and identification of major For t 1, For t 2, For t 3 allergens. *Allergy* .2005 Dec;60(12):1518-23 (SCI)

19. A.P. Bieneman, K.L.Chichester, **Y. H. Chen**, JT. Schroeder. Toll-like Receptor (TLR)2 Ligands Activate Human Basophils for both IgE –dependent and –independent Secretion. *Journal of Allergy and Clinical Immunology*,115(2): 2005 (SCI)

20. 陳弘聖, 唐憶淨,陳怡行,劉丕華. 過敏性反應之診斷及處置. 基層醫學,第二十卷第七期(2005):164-168.

21. Y.M. Chiou, J.L. Lan, T.Y. Hsieh, **Y.H. Chen** and D.Y. Chen. Spontaneous Achillus tendon rupture in a patient with systemic lupus erythematosus due to ischemic necrosis after methylprednisolone pulse therapy. Lupus 14, 321-325. 2005 (SCI)

22. P.S. Creticos, **Y. H. Chen** and J.T. Schroeder. New Approaches of Immunotherapy: Allergen Vaccination with Immunostimulatory DNA. *Immunol Allergy Clin North Am. 2004*;Nov, 24(4):569-81. (SCI)

23. M. F. Lee, **Y. H. Chen**, J. L. Lan, C. Y. Tseng, and C. H. Wu. Allergenic components of Indian jujube (Zizyphus mauritiania) show IgE cross-reactivity with latex allergens. *Int Arch Allergy Immunol* ,133:211-216,2004 (SCI)

24. D.Y. Chen, J.L. Lan, T.Y. Hsieh, **Y.H.Chen**. Clinical manifestations, disease course, and complications of adult-onset Still's disease in Taiwan. *J Formos Med Assoc*. 103(11):844-52.2004(SCI)

25. J. L. Lan, S. J Chou, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and M. Young. A comparative study of etanercep/methotrexate and placebe/methotrexate in Taiwanese patients with active rheumatoid arthritis: A 12-week, double-blind, randomized, placebo-controlled study. *J Formos Med Assoc*,103(8):618-23,2004.(SCI).

26. **Y. H. Chen**, A. P. Bieneman, P. S. Creticos, K. L. Chichester, and J. T. Schroeder. Interferon-alpha inhibits IL-3 priming of human basophil cytokine secretion but not leukotriene C4 and histamine release. *Journal of Allergy and Clinical Immunology*, 112:944-50,2003 (SCI)

27. **Y. H. Chen** and J. L. Lan. Latex allergy and latex-fruit syndrome among medical workers in Taiwan. *J Formos.Med.Assoc.* 101 (9):622-626, 2002.(SCI)

28. S. J. Wang, J. L. Lan, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Effects of cisapride on colonic transit in patients with progressive systemic sclerosis. *Clin Rheumatol.* 21 (4):271-274, 2002.(SCI)

29. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, C. H. Kao, and W. Y. Lin. Solid-phase radionuclide esophageal transit in progressive systemic sclerosis. *Hepatogastroenterology* 49 (46):989-991, 2002.(SCI)

30. S. J. Wang, J. L. La, D. Y. Chen, **Y. H. Chen,** T. Y. Hsieh, and W. Y. Lin. Effects of cisapride on oesophageal transit of solids in patients with progressive systemic sclerosis. *Clin Rheumatol.* 21 (1):43-45, 2002.(SCI)

31. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Solid phase radionuclide esophageal transit in mixed connective tissue disease. *Abdom.Imaging* 27 (1):6-8, 2002.(SCI)

32. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Colonic transit disorders in systemic sclerosis. *Clin Rheumatol.* 20 (4):251-254, 2001.(SCI)

33. **Y. H. Chen** and J. L. Lan. Kikuchi disease in systemic lupus erythematosus: clinical features and literature review. *J Microbiol.Immunol Infect.* 31 (3):187-192, 1998.

會議論文

1. Y. H. Chen , M.F. Lee, S.H. Chang, G.Y. Hwang. Molecular Cloning and characterization of a major Forcipomyia taiwana (biting midge) Allergen For t 2.0101. 2008 FIMSA
2. Y. H. Chen , H.C. Chang, C. Wang, D.Y. Chen. Comparison of Two Calibration Methods for the Automated Enzyme-Linked Immunoassay to Detect Antibodies against Double-Stranded DNA in Serum . 2008 Annual Meeting of Taiwan Rheumatology Association, R.O.C.
3. Y. H. Chen , M.F. Lee, S.H. Chang, G.Y. Hwang. Molecular cloning of a 24kDa Forcipomyia taiwana (biting midge) allergen with sequence homology to trypsin precursor. 2008 Annual Meeting of America Academy of Asthma, Allergy and Immunology. Philadelphia, USA.
4. Y. H. Chen , H.J. Wu, M.F. Lee, G.Y. Hwang. Crude *Foricpomyia taiwana* (biting midge) extracts stimulate PBMC proliferation, IFN-gamma, IL-6 and IL-10 secretion from midge-allergic subjects. 2007 World Allergy Congress. Bankok, Thailand.

5. Y. H. Chen , H.J. Wu, D.Y.Chen, W.N. Huang, J.J. Tsai, J.L. Lan. Autologous Serum Skin Test among Patients with Chronic Urticaria in Taiwan — Frequency and Clinical Relevance , 2006; *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*
6. Y. H. Chen, M.F. Lee, J.L. Lan, G.Y. Hwang, C.H. Wu. Preliminary characterization of a major *Forcipomyia taiwana*(biting midge) allergen. Journal of the World Allergy Organization. 2005; Sppl. No. 1, P186
7. J. T. Schroeder, Y. H. Chen, A. P. Bieneman, K. L. Chichester,E. Siekierski. Relative Expression of Toll-Like Receptor (TLR)-9 Protein in Human Basophils vs. Plasmacytoid Dendritic Cells (PDC) Effects of CpG-ODN on Fc RI mRNA levels. *Journal of Allergy and Clinical Immunology*, 2004;113(2):S102
8. Y. H. Chen, D. Y. Chen, and J. L. Lan. Childhood Primary Sjogren syndrome in Taiwan. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 2000.
9. Y. H. Chen, D. Y. Chen, T. Y. Hsieh, and J. L. Lan. Antineutrophil Cytoplasmic Antibodies in Systemic Lupus Erythematmatosus. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1999.
10. D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Childhood Still's Disease with Brainstem Lesions: reversible MRI Findings After Corticosteroids Therapy. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
11. D. Y. Chen, Y. H. Chen, T. Y. Hsieh, and J. L. Lan. Central Nervous System Manifestation of Primary Sjogren Syndrome. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.* , 1999.
12. D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Hemolytic Uremic Syndrome Complicating Adult-onset Still's Disease: Successful Treatment with Plasma Infusion and Steroid. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
13. J. L. Lan, Y. H. Chen, D. Y. Chen, and T. Y. Hsieh. Canine Systemic Lupus Erythematosus . *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
14. J. L. Lan, Y. H. Chen, and F. Z. Lin. Palindromic rheumatism in Chinese: A long term followed-up and immunogenetic study. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
15. Y. N. Hsu, D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Hashimoto's encephalopathy Complicating Systemic Lupus Erythematosus :Immunological Abnormalities in CSF and MRI Findings. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

16. Y. C. Hsieh, Y. H. Chen, T. Y. Hsieh, D. Y. Chen, and J. L. Lan. Plant Thorn synovitis. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999
17. T. Y. Hsieh, Y. H. Chen, D. Y. Chen, and J. L. Lan. Primary Sjogren syndrome with protein-losing enteropathy. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
18. K. Y. Huang, T. Y. Hsieh, Y. H. Chen, J. L. Lan, and D. Y. Chen. Rheumatoid Pachymeningitis: Immunological Abnormalities of CSF and Reversible MRI Findings. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.
19. Y. H. Chen, J. L. Lan, and T. Y. Hsieh. Latex Allergy in Medical Workers : An Important but overlooked problem in Taiwan. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1998.
20. Y. H. Chen and J. L. Lan. POEMS syndrome. *Local Conference, The Taiwan Society of Internal Medicine,Taichung, Taiwan,R.O.C.*, 1998.
21. Y. H. Chen, J. L. Lan, S. C. Hsieh, and B. C. Shu. Mononeuritis Multiplex in Systemic Lupus Erythematosus. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1997.
22. J. L. Lan, Y. H. Chen, W. Y. Lin, and S. J. Wang. Lupus Nephritis : A Comparison between Laboratory Data, Gallium Image and Renal Biopsy Measuremen. *Joint Meeting of the Chinese Society of Immunology and Rheumatology Association, Taiwan,R.O.C.*, 1997.
23. J. L. Lan, Y. H. Chen, W. Y. Lin, and S. J. Wang. Value of Gallium Renal Scintigraphy in Monitoring the Activity of Lupus Nephritis. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1997.
24. Y. H. Chen and J. L. Lan. Cyclophosphamide-Induced Ovarian Failure in Patient with Lupus Nephritis. *The Seventh Conference of Asian Pacific League of Anti-rheumatism, Malaysia*, 1994.
25. Y. H. Chen, S. J. Chou, J. P. Su, N. S. Lai, and J. L. Lan. HLA-B27 Typing Using Flow Cytometry, Micro-lymphocytotoxicity Test and PCR: A Comparison. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.
26. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Systemic Lupus Erythematosus Associated with Kikuchi Disease. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.
27. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Positron Emission Tomography Scan in Systemic Lupus Erythematosus with CNS Involvement: The First Case

Report in Taiwan. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.

28. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Catastrophic Antiphospholipid Antibody Syndrome in Systemic Lupus Erythematosus : Report of An Autopsy Case. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.

著作

1.OSCE 臨床技術手冊　2007 , 合記書局出版

2.風濕病的理學檢查教學影帶, 2000, 台灣免疫風濕疾病關懷協會

臨床試驗

1. 2008-2012 年　　協同主持人

SERAPHIN: Study with Endothelin Receptor Antagonist in Pulmonary arterial Hypertension to Improve clinical outcome.

2. 2008 年　　協同主持人

An Open-Label Phase I/II Study to Assess the Safety , Tolerability and Efficacy of Three Doses of TuNEX in Patients with Rheumatoid arthritis

3. 2008 年　　協同主持人

A randomized , double-blind, placebo-controlled, multicentre prospective dose-finding Phase II/III study with atacicept given subcutaneously to subjects having recently experienced a flare of systemic lupus erythematosus(SLE). (the APRIL SLE Trial)

4. 2008 年　　協同主持人

Ultracet Combination Tablets in the Treatment of the Pain of Fibromyalgia

5. 2007-2008 年　　協同主持人

A Phase 3, Multi-Center, Randomized, Double-Blind, Placebo-Controlled, 52-Week Study to Evaluate the Efficacy and Safety of Belimumab (HGS1006, LymphoStat-B), a Fully Human Monoclonal Anti-BLys Antibody, in subjects with Systemic Lupus Erythematosus (the BLISS Trial)

6. 2007 年　　協同主持人

Prospective Randomized Evaluation of Celeboxib Integrated Safety vs. Osteoarthritis or Rheumatoid Arthritis Patients With or at High Risk for Cardiovascular Disease

Comparing Celecoxib with Naproxen and Ibuprofen (The PRECISION Trial)

7. 2007 年 協同主持人

A randomized, double-blind, parallel group, international study to evaluate the safety and efficacy of ocrelizumab in combination with methotrexate (MTX) compared to MTX alone in methotrexate-naïve patients with active rheumatoid arthritis

8. 2007 年 協同主持人

A randomized, double-blind, parallel group, international study to evaluate the safety and efficacy of ocrelizumab compared to placebo in patients with active rheumatoid arthritis continuing methotrexate treatment

9. 2007 年 協同主持人

A randomized, multicenter, open-label, 6-month study to explore efficacy and safety of enteric-coated mycophenolate sodium (myfortic) in combination with two corticosteroid regimens for the treatment of lupus nephritis flare(MyLupus Trial)

10. 2007 年 協同主持人

Open-label Study of MabThera (rituximab) in patients with active rheumatoid arthritis who have had an inadequate response or intolerance to one or more anti-TNF agents

11. 2005 年 計畫主持人

A Randomized, Comparative, Parallel, Single-Blinded Study to Compare the Efficacy and Safety between Single-Inhalor and Traditional Final Dosing in Patients with Persistent Asthma

12. 2002 年 計畫主持人

Efficacy and Safety of Sublingual Immunotherapy with House Dust Mite Extract in Children with Allergic Rhinitis: A Multicenter, Double-blind, Randomized, Placebo-controlled Study

13. 2002 年 協同主持人

An International Multicenter, stratified, randomized, double-blind. Double dummy, parallel-group, 52-week gastrointestinal clinical safety to demonstrate that COX189 (400mg QD) reduces the risj to develop complicated ulcers as compared to NSAIDs (naproxen 500mg bid and ibuprofen 800mg tid) in Osteoarthritis patients

14. 2001 年 協同主持人

A Double Blind, Placebo-Controlled,Randomized Study of Pilocarpine for the

Treatment of Xerostomia in Patients with Sjogren Syndrome

15. 2000 年　　協同主持人

A Double Blind, Randomnized, Comparative Placebo-Controlled Study on the Efficacy and Safety of Methotrexate + Etanercept versus Methotrexate + Placebo in Patients with Active Rheumatoid Arthritis

16. 2000 年　　協同主持人

A Multi-center, Double-Blind Parallel Group Study Comparing the Efficacy and Safety Between Leflumedil and Methotrexate in Patients with Rheumatoid Arthritis

17. 1999 年　　協同主持人

18. A Multi-center, Double-Blind Parallel Group Study Comparing the Efficacy and Incidence of Gastroduodenal Ulcer Associated with SC-58635 100mg BID with that of Diclofenac 50mg BID Taken for 12 Weeks in Patients with Osteoarthritis or Rheumatoid Arthritis

19. 1998 年　　計畫主持人

A Multi-center Open Study to Demonstrate the Safety and Efficacy of Accolate in Patients with Mild to Moderate Asthma

20. 1998 年　　協同主持人

21. A phase III Clinical Trial of GL701 in the Management of Female Systemic Lupus Erythematosus Patients in Taiwan

22. 1997 年　　協同主持人

23. A randomized, double-blind, placebo-controlled study comparing icam 7.5mg 與 Diclofenac 100mgin patients with osteoarthritis

Curriculum Vitae

Yi-Hsing Chen, M.D., PhD.

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Current Position 1.Attending Physician

Division of Allergy, Immunology & Rheumatology

Department of Internal Medicine

Taichung Veterans' General Hospital

2. Assistant Professor of Medicine

National Yang-Ming University

3.Clinical Associate Professor of Medicine

National Defense Medical College

4. General Secretary

Rheumatology Association, Taiwan, R.O.C.

Education

2004-2009 PhD degree. Department of Life Science, Tunghai University

	1984-1991	M.D. degree. School of Medicine, China Medical University
Postgraduate Training		
	2008	The Program for Leading Innovations in Health Care and Education, Harvard Macy Institute, Harvard Medical School.
	2004-	PhD program, Department of Life Science, Tunghai University
	2002-2003	Visiting scientist, Johns Hopkins Asthma and Allergy Center. (DNA Vaccine for allergen Immunotherapy)
	1998-present	Attending Physician, Division of Allergy, Immunology & Rheumatology, Taichung Veterans General Hospital
	1997-1998	Fellow, Division of Allergy, Immunology & Rheumatology, Taichung Veterans General Hospital
	1995-1997	Attending Physician, Division of Allergy, Immunology & Rheumatology, Pu-Li Veterans Hospital
	1993-1995	Fellow/Chief Resident, Division of Allergy, Immunology & Rheumatology, Taichung Veterans General Hospital
	1991-1993	Resident, Department of Internal Medicine, Taichung Veterans General Hospital
	1990-1991	Intern, National Taiwan University Hospital
Board Certifications	1. Board of Internal Medicine, Taiwan R.O.C.	
	2. Board of Allergy and Clinical Immunology, Taiwan, R.O.C.	
	3.Board of Rheumatology, Taiwan, R.O.C.	
Professional Organizations	1. American Academy of Allergy, Asthma and Immunology	
	2. Taiwan Society of Internal Medicine, R.O.C.	
	3. Chinese Society of Immunology, Taiwan, R.O.C.	

4. Rheumatology Association, Taiwan, R.O.C.

5. Formosian Medical Association

6. Taiwan Medical Education Association

Research Grants

14. 2006－2009 ,NSC-95-2314-B-075A-016-MY3, Principal investigator, National Science Council, Principal investigator. Standardize the diagnostic method of *Forcipomya taiwana* allergy using recombinant DNA technology
15. 2005, NSC-94-2314-B-075A-001, Principal investigator, National Science Council , Colonization, identification and clinical characterization of *Forcipomya taiwana* allergen(2/2)
16. 2005, TCVGH-943804B : Principal investigator. Identification of *Forcipomya taiwana* allergen using peptide mass fingerprinting.
17. 2004, NSC93-2314-B-075A-006, National Science Council ,2004 , Principal investigator, Colonization, identification and clinical characterization of *Forcipomya taiwana* allergen(1/2)
18. 2004, National Health Bureau ,Department of Health, Principal investigator , A hospital-based Screening Program for Natural Latex Allergy in Medical Workers
19. 2002, NSC 91-2320-B-075A-002: National Science Council, Co-investigator:Cloning and characterization of the latex cross-reacting allergens of an novel fruit Indian jujube (Zizyphus mauritiana)
20. 2002, TCVGH-91913802A: Principal investigator :The Prevalence of Latex-Fruit Syndrome in Taiwan
21. 2001, TCVGH-903802B：Principal investigator:The Prevalence of Natural Rubber Latex Allergy among Atopics and Healthy Blood Donors in Taiwan
22. 1999, TCVGH-883803: Principal investigator : Antineutrophil Cytoplasmic Antibodies in Systemic Lupus Erythematmatosus.

Honors and Awards:

8. 2008, Best teaching faculty of the year, National Yang-Ming University
9. 2008, Best teaching faculty of the year, Taichung Veterans General Hosptial
10. 2006, Best research paper of the year, Taichung Veterans General Hospital
11. 2004, Honorable doctor of the year, Veterans Affairs Commission of the Executive Yuan
12. 2001, Honorable doctor of the year, Taichung City
13. 1996, Honorable doctor of the year, Pu-Li Veterans Hospital
14. 1995, Best Chief Resident, Taichung Veterans General Hospital

Publications

Journal Articles

1. **Y.H. Chen**, M.F. Lee, J.J. Tsai, H.J. Wu,G.Y. Hwang. Specific IgE and IgG responses and cytokine profile in subjects with allergic reactions to biting midge *Forcipomyia taiwana*. *Int Arch Allergy Immunol.* ; 150(1):66-74. 2009 (SCI)
2. **Y.H. Chen**, M.F. Lee, H. J. Wu, J.J. Tsai. Anaphylactic shock to a 33-kDa alpha-S1-casein-like protein in kingfish caviar. *Journal of Investigational Allergology and Clinical Immunology*, 9 (3), May-June 2009 (SCI)
3. T.M. Yu, **Y.H. Chen**, J.Y. Hsu, Y.W. Chuang, C.H. Cheng, C.H. Chen and K.H. Shu Systemic Inflammation is Associated with Pulmonary Hypertension in Patients Undergoing Hemodialysis. *Nephrol Dial Transplant* .Accepted Dec. 2008.(SCI)
4. C. T. Lin, D. Z. Hung, D. Y. Chen, H. J. Wu, J. L. Lan, and **Y. H. Chen***. A hospital-based screening study of latex allergy and latex sensitization among medical workers in Taiwan. *J Microbiol.Immunol Infect.* 41 (6):499-506, 2008.

5. M.F. Lee, J.J. Tsai, G.Y. Hwang, S.J. Lin, **Y.H. Chen***.Identification of immunoglobulin E (IgE)-binding epitopes and recombinant IgE reactivities of a latex cross-reacting Indian jujube Ziz m 1 allergen. *Clin Exp Immunol.* 2008;152(3):464-71 (SCI).

6. T.M. Yu, S.H. Lin, Y.W. Chuang, M.C.Wen, **Y.H. Chen**, C.H. Cheng,C.H. Chen, C.S. Chin and K.H. Shu. A syndrome resembling Bartter's syndrome in sarcoidosis. *Nephrol Dial Transplant* .2008. Nov 5. [Epub ahead of print] (SCI)

7. T.M. Yu, **Y.H. Chen**, J.L.Lan, C.H. Cheng, C.H. Chen, M.J. Wu, K.H. Shu. Renal outcome and evolution of disease activity in Chinese lupus patients after renal transplantation. *Lupus.* ;2008；17(7):687-94.(SCI)

8. T.W. Hseun, D.Y. Chen, T.Y. Hsieh, W.N. Huang, **Y.H. Chen***, J.L. Lan. Hypereosinophilic syndrome in a medical center in Taiwan—an analysis of fourteen cases. *Formosan Journal of Rheumatology* 2008;22:60-67

9. H.K.Lin, J.L.Lan, D.Y. Chen, **Y.H. Chen**, W.N. Huang, T.Y. Hsieh, C.W. Hsieh, H.H. Chen. The diagnostic value of anti-cyclic citrullinated peptide antibodies and rheumatoid factor in patients with rheumatoid arthritis. *Formosan Journal of Rheumatology* 2008;22:68-73

10. 藍忠亮、陳得源、**陳怡行**.新進醫事人員訓練現況與發展—醫院臨床訓練教育的轉變。醫療品質雜誌 2008; 2(6):13-16

11. 翁嘉澤 **陳怡行** 劉明輝. 慢性特發性蕁麻疹. 內科學誌. 2008 年 4 月

12. C.H.Chen, **Y.H. Chen**, J.L.Lan, D.Y. Chen. Scleroderma renal crisis: a rare complication of systemic sclerosis with poor prognosis-experience in a medical center in central Taiwan. *Journal of Rheumatology. R.O.C.* 2007;21:59-64.

13. C.T. Lin, **Y.H. Chen***, D.Y. Chen, W.N. Huang, J.L.Lan. Low dose intravenous cyclophosphamide-induced ovarian failure in Chinese patients with lupus

nephritis. *Journal of Rheumatology. R.O.C*. 2007;21:53-58.

14. C.H.Tung, **Y.H,Chen**, H.H.C. Lan, T.Y. Hsieh, D.Y. Chen, J.L. Lan. Diagnosis of plant thorn synovitis by high-resolution ultrasonography – a case report and literature review *Clin Rheumatol*. 2007,26:849-851.（SCI）
15. K.D. Hong, **Y.H. Chen**, D.Y. Chen, J.L. Lan. Subcutaneous panniculitis-like T-cell lymphoma presenting with hemophagocytic lymphohistiocytic and skin lesions with characteristic high-resolution ultrasonographic findings. *Clin Rheumatol.* 2007,26:775-778（SCI）
16. M.F. Lee, **Y. H. Chen**, H.C. Lin, H.L. Wang, G.Y. Hwang, C.H. Wu. Identification of Hevamine and Hev b1 as major latex allertens in Taiwan. *Int Arch Allergy Immunol* 2006;139(1):38-44. (SCI)
17. M.F. Lee, G.Y. Hwang, **Y.H.Chen,** H.C. Lin, C.H. Wu. Molecular cloning of Indian jujube(Zizyphus mauritiana) Ziz m 1 with sequence similarity to plant class III chitinase. *Molecular Immunology*. 2006, Mar; 43(8):1144-51 (SCI)
18. **Y. H. Chen**, M. F. Lee, J. L. Lan, C.S.Chen, H.L. Lin, G. Y. Huang, and C. H. Wu. Hypersensitivity to Forcipomyia taiwana (biting midge): Clinical analysis and identification of major For t 1, For t 2, For t 3 allergens. *Allergy* .2005 Dec;60(12):1518-23 (SCI)

19. A.P. Bieneman, K.L.Chichester, **Y. H. Chen**, JT. Schroeder. Toll-like Receptor (TLR)2 Ligands Activate Human Basophils for both IgE –dependent and –independent Secretion. *Journal of Allergy and Clinical Immunology*,115(2): 2005 (SCI)

20. 陳弘聖, 唐憶淨,陳怡行,劉丕華. 過敏性反應之診斷及處置. 基層醫學,第二十卷第七期(2005):164-168.

21. Y.M. Chiou, J.L. Lan, T.Y. Hsieh, **Y.H. Chen** and D.Y. Chen. Spontaneous Achillus tendon rupture in a patient with systemic lupus erythematosus due to ischemic necrosis after methylprednisolone pulse therapy. Lupus 14, 321-325. 2005 (SCI)

22. P.S. Creticos, **Y. H. Chen** and J.T. Schroeder. New Approaches of Immunotherapy: Allergen Vaccination with Immunostimulatory DNA. *Immunol Allergy Clin North Am. 2004*;Nov, 24(4):569-81. (SCI)

23. M. F. Lee, **Y. H. Chen**, J. L. Lan, C. Y. Tseng, and C. H. Wu. Allergenic components of Indian jujube (Zizyphus mauritiania) show IgE cross-reactivity with latex allergens. *Int Arch Allergy Immunol* ,133:211-216,2004 (SCI)

24. D.Y. Chen, J.L. Lan, T.Y. Hsieh, **Y.H.Chen**. Clinical manifestations, disease course, and complications of adult-onset Still's disease in Taiwan. *J Formos Med Assoc*. 103(11):844-52.2004(SCI)

25. J. L. Lan, S. J Chou, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and M. Young. A comparative study of etanercep/methotrexate and placebe/methotrexate in Taiwanese patients with active rheumatoid arthritis: A 12-week, double-blind, randomized, placebo-controlled study. *J Formos Med Assoc*,103(8):618-23,2004.(SCI).

26. **Y. H. Chen**, A. P. Bieneman, P. S. Creticos, K. L. Chichester, and J. T. Schroeder. Interferon-alpha inhibits IL-3 priming of human basophil cytokine secretion but not leukotriene C4 and histamine release. *Journal of Allergy and Clinical Immunology*, 112:944-50,2003 (SCI)

27. **Y. H. Chen** and J. L. Lan. Latex allergy and latex-fruit syndrome among medical workers in Taiwan. *J Formos.Med.Assoc.* 101 (9):622-626, 2002.(SCI)

28. S. J. Wang, J. L. Lan, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Effects of cisapride on colonic transit in patients with progressive systemic sclerosis. *Clin Rheumatol.* 21 (4):271-274, 2002.(SCI)

29. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, C. H. Kao, and W. Y. Lin. Solid-phase radionuclide esophageal transit in progressive systemic sclerosis. *Hepatogastroenterology* 49 (46):989-991, 2002.(SCI)

30. S. J. Wang, J. L. La, D. Y. Chen, **Y. H. Chen,** T. Y. Hsieh, and W. Y. Lin. Effects of cisapride on oesophageal transit of solids in patients with progressive systemic sclerosis. *Clin Rheumatol.* 21 (1):43-45, 2002.(SCI)

31. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Solid phase radionuclide esophageal transit in mixed connective tissue disease. *Abdom.Imaging* 27 (1):6-8, 2002.(SCI)

32. S. J. Wang, J. L. Lan, D. Y. Chen, **Y. H. Chen**, T. Y. Hsieh, and W. Y. Lin. Colonic transit disorders in systemic sclerosis. *Clin Rheumatol.* 20 (4):251-254, 2001.(SCI)

33. **Y. H. Chen** and J. L. Lan. Kikuchi disease in systemic lupus erythematosus: clinical features and literature review. *J Microbiol.Immunol Infect.* 31 (3):187-192, 1998.

Conference Papers

1. Y. H. Chen , M.F. Lee, S.H. Chang, G.Y. Hwang. Molecular Cloning and characterization of a major Forcipomyia taiwana (biting midge) Allergen For t 2.0101. 2008 FIMSA
2. Y. H. Chen , H.C. Chang, C. Wang, D.Y. Chen. Comparison of Two Calibration Methods for the Automated Enzyme-Linked Immunoassay to Detect Antibodies against Double-Stranded DNA in Serum . 2008 Annual Meeting of Taiwan Rheumatology Association, R.O.C.
3. Y. H. Chen , M.F. Lee, S.H. Chang, G.Y. Hwang. Molecular cloning of a 24kDa Forcipomyia taiwana (biting midge) allergen with sequence homology to trypsin precursor. 2008 Annual Meeting of America Academy of Asthma, Allergy and Immunology. Philadelphia, USA.
4. Y. H. Chen , H.J. Wu, M.F. Lee, G.Y. Hwang. Crude *Foricpomyia taiwana* (biting midge) extracts stimulate PBMC proliferation, IFN-gamma, IL-6 and IL-10 secretion from midge-allergic subjects. 2007 World Allergy Congress. Bankok, Thailand.

5. Y. H. Chen , H.R. Wu, D.Y.Chen, W.N. Huang, J.J. Tsai, J.L. Lan. Autologous Serum Skin Test among Patients with Chronic Urticaria in Taiwan — Frequency and Clinical Relevance , 2006; *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*

6. Y. H. Chen, M.F. Lee, J.L. Lan, C.H. Wu. Preliminary characterization of a major Forcipomyia taiwana(biting midge) allergen. Journal of the Whord Allergy Organization. 2005; Sppl. No. 1, P186

7. J. T. Schroeder, Y. H. Chen, A. P. Bieneman, K. L. Chichester,E. Siekierski. Relative Expression of Toll-Like Receptor (TLR)-9 Protein in Human Basophils vs. Plasmacytoid Dendritic Cells (PDC) Effects of CpG-ODN on FcεRIα mRNA levels. *Journal of Allergy and Clinical Immunology*, 2004;113(2):S102

8. Y. H. Chen, D. Y. Chen, and J. L. Lan. Childhood Primary Sjogren syndrome in Taiwan. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 2000.

2. Y. H. Chen, D. Y. Chen, T. Y. Hsieh, and J. L. Lan. Antineutrophil Cytoplasmic Antibodies in Systemic Lupus Erythematmatosus. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1999.

3. D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Childhood Still's Disease with Brainstem Lesions: reversible MRI Findings After Corticosteroids Therapy. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

4. D. Y. Chen, Y. H. Chen, T. Y. Hsieh, and J. L. Lan. Central Nervous System Manifestation of Primary Sjogren Syndrome. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.* , 1999.

5. D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Hemolytic Uremic Syndrome Complicating Adult-onset Still's Disease: Successful Treatment with Plasma Infusion and Steroid. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

6. J. L. Lan, Y. H. Chen, D. Y. Chen, and T. Y. Hsieh. Canine Systemic Lupus Erythematosus . *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

7. J. L. Lan, Y. H. Chen, and F. Z. Lin. Palindromic rheumatism in Chinese: A long term followed-up and immunogenetic study. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

8. Y. N. Hsu, D. Y. Chen, T. Y. Hsieh, Y. H. Chen, and J. L. Lan. Hashimoto's

encephalopathy Complicating Systemic Lupus Erythematosus :Immunological Abnormalities in CSF and MRI Findings. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

9. Y. C. Hsieh, Y. H. Chen, T. Y. Hsieh, D. Y. Chen, and J. L. Lan. Plant Thorn synovitis. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999

10. T. Y. Hsieh, Y. H. Chen, D. Y. Chen, and J. L. Lan. Primary Sjogren syndrome with protein-losing enteropathy. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

11. K. Y. Huang, T. Y. Hsieh, Y. H. Chen, J. L. Lan, and D. Y. Chen. Rheumatoid Pachymeningitis: Immunological Abnormalities of CSF and Reversible MRI Findings. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1999.

12. Y. H. Chen, J. L. Lan, and T. Y. Hsieh. Latex Allergy in Medical Workers : An Important but overlooked problem in Taiwan. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1998.

13. Y. H. Chen and J. L. Lan. POEMS syndrome. *Local Conference, The Taiwan Society of Internal Medicine,Taichung, Taiwan,R.O.C.*, 1998.

14. Y. H. Chen, J. L. Lan, S. C. Hsieh, and B. C. Shu. Mononeuritis Multiplex in Systemic Lupus Erythematosus. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association,Taiwan,R.O.C.*, 1997.

15. J. L. Lan, Y. H. Chen, W. Y. Lin, and S. J. Wang. Lupus Nephritis : A Comparison between Laboratory Data, Gallium Image and Renal Biopsy Measuremen. *Joint Meeting of the Chinese Society of Immunology and Rheumatology Association, Taiwan,R.O.C.*, 1997.

16. J. L. Lan, Y. H. Chen, W. Y. Lin, and S. J. Wang. Value of Gallium Renal Scintigraphy in Monitoring the Activity of Lupus Nephritis. *Joint Meeting of the Chinese Society of Immunology & Rheumatology Association, Taiwan,R.O.C.*, 1997.

17. Y. H. Chen and J. L. Lan. Cyclophosphamide-Induced Ovarian Failure in Patient with Lupus Nephritis. *The Seventh Conference of Asian Pacific League of Anti-rheumatism, Malaysia*, 1994.

18. Y. H. Chen, S. J. Chou, J. P. Su, N. S. Lai, and J. L. Lan. HLA-B27 Typing Using Flow Cytometry, Micro-lymphocytotoxicity Test and PCR: A Comparison. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.

19. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Systemic Lupus Erythematosus Associated with Kikuchi Disease. *Annul Meeting of Chinese*

Society of Immunology,Taiwan,R.O.C., 1994.

20. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Positron Emission Tomography Scan in Systemic Lupus Erythematosus with CNS Involvement: The First Case Report in Taiwan. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.

21. Y. H. Chen, C. P. Chang, D. Won, and J. L. Lan. Catastrophic Antiphospholipid Antibody Syndrome in Systemic Lupus Erythematosus : Report of An Autopsy Case. *Annul Meeting of Chinese Society of Immunology,Taiwan,R.O.C.*, 1994.

Book publication

1. OSCE Clinical Skills Handbook, 2007

2. Rheumatologic examination, 2000

Clinical Trials

2008-2012 Co-investigator

SERAPHIN: Study with Endothelin Receptor Antagonist in Pulmonary arterial Hypertension to Improve clinical outcome.

2008 Co-investigator

An Open-Label Phase I/II Study to Assess the Safety , Tolerability and Efficacy of Three Doses of TuNEX in Patients with Rheumatoid arthritis

2008 Co-investigator

A randomized , double-blind, placebo-controlled, multicentre prospective dose-finding Phase II/III study with atacicept given subcutaneously to subjects having recently experienced a flare of systemic lupus erythematosus(SLE). (the APRIL SLE Trial)

2008 Co-investigator

Ultracet Combination Tablets in the Treatment of the Pain of Fibromyalgia

2007-2008 Co-investigator

A Phase 3, Multi-Center, Randomized, Double-Blind, Placebo-Controlled, 52-Week Study to Evaluate the Efficacy and Safety of Belimumab (HGS1006, LymphoStat-B), a Fully Human Monoclonal Anti-BLys Antibody, in subjects with Systemic Lupus

Erythematosus (the BLISS Trial)

2007 Co-investigator

Prospective Randomized Evaluation of Celeboxib Integrated Safety vs. Osteoarthritis or Rheumatoid Arthritis Patients With or at High Risk for Cardiovascular Disease Comparing Celecoxib with Naproxen and Ibuprofen (The PRECISION Trial)

2007 Co-investigator

A randomized, double-blind, parallel group, international study to evaluate the safety and efficacy of ocrelizumab in combination with methotrexate (MTX) compared to MTX alone in methotrexate-naïve patients with active rheumatoid arthritis

2007 Co-investigator

A randomized, double-blind, parallel group, international study to evaluate the safety and efficacy of ocrelizumab compared to placebo in patients with active rheumatoid arthritis continuing methotrexate treatment

2007 Co-investigator

A randomized, multicenter, open-label, 6-month study to explore efficacy and safety of enteric-coated mycophenolate sodium (myfortic) in combination with two corticosteroid regimens for the treatment of lupus nephritis flare(MyLupus Trial)

2007 Co-investigator

Open-label Study of MabThera (rituximab) in patients with active rheumatoid arthritis who have had an inadequate response or intolerance to one or more anti-TNF agents

2005 Principle investigator

A Randomized, Comparative, Parallel, Single-Blinded Study to Compare the Efficacy and Safety between Single-Inhalor and Traditional Final Dosing in Patients with Persistent Asthma

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Efficacy and Safety of Sublingual Immunotherapy with House Dust Mite Extract in Children with Allergic Rhinitis: A Multicenter, Double-blind, Randomized, Placebo-controlled Study

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