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行政院衛生署疾病管制局九十五年度科技研究發展計畫

研發以新型醣脂佐劑結合抗人類流感及禽流感之雙效疫苗

研 究 報 告

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摘要

(1)中文摘要

本計畫預期研發有效對抗人類流感及禽流感的雙效疫苗。何大一院士的研究團隊已經根據流感病毒血凝素 HA 基因及病毒基質 M2 蛋白基因設計抗 H5N1 流感病毒的 2 種 DNA 疫苗，二者在體外實驗上均能大量表現。對小鼠施用抗 HA DNA 疫苗，可誘發完整的免疫保護效能，不受致命劑量禽流感疫苗株 NIBRG-14 感染的影響,且可能對不同演化枝 H5N1 禽流感病毒都有交叉保護的作用。翁啟惠院士的團隊大量合成 alpha-GalCer (C1) 醣脂，進行免疫調節效力的動物試驗。並已設計研製 16 種 C1 的類似物。陳鈴津博士的團隊以其中 17 種醣脂類似物進行體外實驗，結果顯示在 acryl 末端 (第三族) 或 spingosine 末端 (第四族) 含有芳香環的醣脂類似物，如 C11、C13、及 C16，在激發 Th1 細胞激素/趨化素 (cytokines /chemokines) 表現、T 細胞受體活化、及人類自然殺手 T 細胞增殖等作用，比原型 C1 醣脂還更有效。在小鼠活體試驗中，以醣脂激發激發細胞激素與趨化素表現的強度高低，投藥途徑依序為：靜脈注射 > 肌肉注射 > 皮下注射。此外，我們也發現 C1 及 C11 醣脂曾被用來作為破傷風類毒素的強效佐劑。總體來說，這些發現對 H5N1 疫苗的開發提供了重要的臨床參考。

生物技術開發中心則建立抗病毒疫苗製作平台技術，以承接抗病毒疫苗後續之開發，包括疫苗放大合成、配方研究、及毒理試驗等。目前，生物技術開發中心利用已構築之愛滋病疫苗 ADVAX 載質體進行生產流程的建立及試製，已將製程放大至 20 公升的規模，回收純化的過程中，能有效地去除宿主 RNA 和宿主蛋白質，plasmid DNA 的回收率和

supercoiled form plasmid 的比例皆可達到工業生產的水準，未來將繼續將製程規模擴大到 50~100 公升的醱酵規模。

中文關鍵詞：禽流感、次單元疫苗、醣脂、佐劑、DNA 疫苗

(2) **英文摘要**

This project is aiming to develop a bivalent influenza vaccine against avian and human influenza A virus. Dr. Ho's group has designed HA- and M2-based DNA vaccines against H5N1 viruses and both vaccines have good expression level in vitro. The HA-based vaccine can induce full protective immunity for a lethal dose of heterologous H5N1 mucosal route challenge. It may also confer protection across H5N1 of different clades. Dr. Wong's group has synthesized alpha-GalCer (C1) in sufficient quantities for animal studies. His group also designed and developed 16 analogs of C1. Dr. Yu's group examined the in vitro activation of NKT cells by the 17 C1 analog compounds and showed that glycolipids containing an aromatic ring in their acyl tail (group III) or spingosine tail (group IV), esp. C11, C13 and C16 were more effective than α-GalCer in inducing Th1 cytokines/chemokines, TCR activation and human NKT expansion. In vivo studies in mice revealed that among three routes of administration, the order of potency in cyokine induction was I.V.>I.M.>S.C. We also found that both C1 and C11glycolipids acted as potent adjuvants for tetanus toxoid vaccine. Taken together, these findings have major clinical implications for H5N1 vaccine development.

The Development Center for Biological (DCB) has establishes the platform of DNA vaccine for virus, and will continue the development of anti-viral

DNA vaccine candidate, including mass-production, formula research, and preclinical toxicology experiments. At present, the DCB is using ADVAX HIV vaccine provided by David Ho to design the standard protocol. The scale has now been enlarged to 20-liter. The E. coli host RNA and the proteins were removed effectively in the plasmid purification process. The recovery of plasmid DNA and the proportion of supercoiled form plasmid agreed with the industrial requirement. In the future, the fermentation scale will be expanded to 50~100-liter for the mass production of DNA vaccines.

Keyword: avian influenza, subunit vaccine, glycolipid, adjuvant, DNA vaccine

本文

(1)前言

許多新興或捲土重來的流行性疾病已成為威脅人類健康的迫切問題，嚴重急性呼吸道症候群 (SARS)、流行性感冒及禽流感病毒就是這幾年來全球公共衛生及健康的頭條新聞；其它病毒感染，如 71 型腸病毒 (Enterovirus)、馬堡病毒 (Marburg virus)、登革熱病毒 (Dengue virus)、愛滋病毒 (HIV)、克沙奇病毒 (Coxsackie virus) 等也嚴重威脅到人類健康。

近幾年來多個亞洲國家報告雞隻中出現禽流感，主要影響家禽，但人類亦有受感染的個案。未經過豬而直接感染人類的禽流感病毒目前所知的有 H5N1、H7N7 及 H9N2 等型。H5N1 禽流感病毒於 1961 年於南非分離，1997 年香港暴發 H5N1 禽流感流行，此次流行造成 18 人感染，6 人死亡，以及無數家禽的死亡直接由家禽傳給人類。2001 年禽流感再次襲擊香港，造成大量雞隻死亡。2003 年起世界各地更陸續傳出禽流感。

H5N1 禽流感病毒是 A 型流感的一種，本來只影響禽類，如雞、鴨等，而香港在 1997 及 2003 年分別發現有 18 宗及 2 宗人類感染的個案。H5N1 禽流感是透過與活家禽近距離接觸或由受感染的禽類或其排泄物而感染禽流感，而人類之間的傳播能力十分低。H5N1 禽流感的徵狀與普通流感差不多，包括發燒、全身肌肉疼痛、咳嗽和喉嚨痛，但較易導致高燒、肺炎、呼吸衰竭、多種器官衰竭，以致死亡。

國際衛生組織甚至發出聲明，2006 年有可能爆發全球禽流感大流行，而一旦禽流感傳染途徑變成人傳人，以及流感病毒變種，全球將可能造成最少 700 萬到上億人喪生，死亡率更高達百分之五十之多。目前世界各國都極力囤積感冒藥和感冒疫苗。

每幾年都會爆發一次大流行的流行性感冒，與甚至可能侵襲人類的禽

流感的情況，已使全球意識到建立疫苗研發機構的重要性。例如日本最近在東京成立一病毒學研究中心，致力於流行性疾病的研究，而臺灣長久以來對於病毒的相關研究本就非常活躍，尤其是肝炎病毒 (hepatitis virus)、非洲淋巴細胞瘤病毒 (EBV) 及登革熱病毒等。目前國內有相當多病毒學家散佈在各大學或研究機構，其中在中央研究院在各研究所也有將近 20 位病毒學家。然而在特定的研究領域，例如流行性感冒及其治療策略之發展，就沒有相關研究人員，於是臺灣始終無法掌握流行性感冒的疫情並加以控制。

因此我們憂心，未來世界性流行性感冒一旦爆發，或是禽流感病毒突變成人與人之間的傳染，屆時各國將自顧不暇，而我們國家如果沒有能力自己製造疫苗及藥物，又無法從其他地方獲得這些防治流感的工具，則將會再遭類似 SARS 的慘痛經歷。因此進行病毒研究及其發展控制之藥物及疫苗，的確為目前最重要，且有燃眉之急的公共衛生課題。

以目前中研院的研究能力，在對致病基因之發現、結構與功能生物學、化學生物學、幹細胞、癌細胞、病毒、細菌及免疫細胞之研究，已有相當成果。基因體研究中心翁啟惠院士所領導建立的新藥研發團隊研究，對於流行性感冒及禽流感的研究及相關藥物開發有絕佳優勢。而何大一院士在全球愛滋病的治療研發向來居領導地位，他利用愛滋病毒 SF162 Delta V2 鞘蛋白成功製備 DNA 疫苗 1。而在 SARS 疫情爆發之後，他更將其冠狀病毒 spike 醣蛋白與細胞受器的結合區域重組於疫苗載體，也可以引發中和抗體的產生，達到保護作用 2。翁啟惠院士同時在醣類研究為國際權威，研究醣脂對於調節 NKT 細胞免疫作用的效能，及增加免疫功能，也都有突破性的進展 3-5。陳鈴津博士則是國內知名的轉譯醫學專家，同時在腫瘤的免疫治療也累積了相當的經驗。她曾經研發抗 neuroblastoma 與 osteosarcoma 的單株抗體，並完成臨床第一期與第二期試驗 6。

綜觀以上利基，中央研究院基因體研究中心由翁啟惠院士所建立的新藥研發研究成果，加上何大一院士對疫苗研發的經驗，以及陳津鈴博士對臨床前及臨床試驗之設計及執行之經驗，將可使流感、禽流感疫苗的研發能更順遂、並縮短時程，將來更能將研究成果在最短的時間內發展為醫療用藥。本跨領域整合計畫，將由實驗室的基礎研究成果，延展至疫苗技術之研發及應用，最終目標在於希望可以研發及製造安全的流感及禽流感疫苗，不僅可以提昇國內流行病防疫工作，亦可解決目前全球最重要之公共衛生問題；而更實質的利益在於，與國人共享研發成果，供給國人流感、禽流感疫苗所需，國產國用，不必再仰賴國外進口，並能使我國建立完整防範新興突發傳染病的設備與能力，增進國民健康之福祉，減少如 SARS 引起的社會成本的損失，甚至還能出口至其他有需要的國家，為促進全人類健康福祉盡一份力。

(2)材料與方法

目前已知 A 型流感病毒有十一個蛋白，其中：血凝素 (hemagglutinin) 和神經胺酸酶 (neuraminidase) 可以做抗流感藥物的標的。目前在可用的抗流感藥物中，克流感 (Tamiflu) 及 Relenza 便是抗神經胺酸酶的藥物。由於病毒的易變性，如今已有對抗神經胺酸酶藥物有抗藥性的流感病毒在流傳中；因此我們必須開發新的技術來制服這些對當今的藥物有抗藥性的新病毒。在本計畫中將合成一雙效 M2/H5 疫苗，以對抗禽流感或其他 A 型流感病毒，並輔以可引發免疫反應的醣脂做為佐劑以加強疫苗的效果。

經由疫苗研發所構築的含有不同標的基因的 DNA 疫苗，在發展醫療及 GMP 規格的製程技術時，需考慮所使用的大腸桿菌 (E. coli) 宿主細胞、表現載體 (骨架的大小及外來基因容量的大小)、特定菌株的單位產量 (mg

DNA/g 菌液濕重)。以醱酵槽可利用大腸桿菌高密度醱酵製程培養技術來提高 DNA 的產量，依宿主細胞、載體及使用基因之大小，所能得到的產量在 50-200 mg DNA/公升。而後續則需要用大型摩過濾回收設備濃縮菌體，並進行鹼性溶菌法，去除細胞碎片段，進行濃縮前處理，再利用數步的管柱層析達到醫藥級的規格，並需針對所定的規格發展品管方法，通過檢驗後才能放行使用。

A. 以疫苗載體製備疫苗

由於何大一院士所發展以疫苗載體表現標的抗原的次單元疫苗技術，已成功地引發對抗愛滋病毒的免疫反應，現在美國都已進入臨床試驗階段。DNA 載體具有 2 啟動子，pCMV 及 phEF1a，可表現 2 不同的外來基因，此 DNA 疫苗在經由電穿孔方式送入細胞後可引發極強的免疫反應；MVA 載體則具有可以插入多個外來基因的強大包容性。本計畫將沿用同樣的策略，利用疫苗載體重組 H5 及 M2 的部分序列成為融合基因。現在已知的各種 H5 病毒變異度高達 7%，而其他具高保留度的序列應該是 H5 病毒所必需的序列，將可以涵蓋如 H5N1 病毒株或將來可能竄起的變種病毒的必需序列，成為極佳的攻擊對象。但有時爆發大流行性感冒的病毒株可能因變異較大，而避開這樣的疫苗免疫反應。因此我們設計在同一載體上再插入另一段 A 型流感病毒 M2 (Ion channel pretein) 蛋白的共有序列，可加強疫苗的免疫作用，同時也可針對 A 型流感施用。疫苗載體的組成包括：前導序列 (leader sequence，帶領所表現的融合蛋白質分泌至細胞外)、免疫球蛋白 G 的 Fc 片段 (將抗原帶領至抗原呈現細胞 (antigen presenting cell) 的 Fc 受器、通用輔助 T 細胞的抗原決定部位、以及 CD40L 融合序列盒 (傳遞抗原至樹突細胞 (dendritic cell)，並且活化樹突細胞使之成熟)。

第一年：

1. M2 多基因片段融合疫苗的設計、合成、M2 蛋白質的表現與純化

   尋找並確認 M2 蛋白之外露區域序列

   合成 M2 蛋白外露區域之 DNA 序列片段融合基因

   將融合基因插入疫苗載體

   疫苗蛋白質的表現與純化

2. H5 血凝素疫苗的設計、合成、H5 蛋白質的表現與純化

   比對 H5 型與東南亞型禽流感病毒血凝素的高保留度序列

   合成血凝素之高保留度 DNA 序列片段融合基因

   將融合基因插入疫苗載體

   疫苗蛋白質的表現與純化

   測試免疫血清對於細胞表面上 HA 蛋白的結合力

3. 生產 H5 DNA 疫苗，進行小鼠免疫原性試驗，並生產α-Gal-Cer 以測試α-Gal-Cer 輔助 H5 疫苗的佐劑效能

B. 製備及篩選新型醣脂作為疫苗的佐劑

計畫合成超過二十種不同醣脂，並測試其對人體 NKT 細胞之活性，從 IFN-γ及 IL-4 產生之比例，選擇適當的醣脂當佐劑，以供進一步在小鼠進行單獨試驗或與研發之疫苗一同試驗。從其抗體產生之免疫反應及對抗 H5N1 病毒之效果，來決定疫苗與醣脂之最佳組合，以供進一步之動物試驗。

第一年：

1. 設計並合成醣脂

   根據結構、活性與免疫學之知識，設計並合成 20 種以上不同醣脂

2. 篩選醣脂

以細胞結合、細胞激素活化能力篩選醣脂候選物

(3) 結果與討論

一、DNA 疫苗與以新型醣脂作為疫苗佐劑的研發

A. Design of H5- and M2-based DNA vaccines

There are more than 500 hemagglutinin genes of H5N1 virus available in the database. The biggest problem encountered during H5N1 vaccine development is the cross-protection issue. For example, the Vietnam vaccine strain could induce full protection against itself, but fails to induce protective immunity against an Indonesia H5N1 strain. Therefore, in order to cover the genetic variability and thus induce cross-protection across different H5N1 strains, we deduced a consensus HA sequence from HA gene of 500 H5N1 virus strains and used this consensus sequence for vaccine development effort. As shown in Fig. 1a, our designed consensus HA is located between Indonesia H5 and Vietnam H5 in the phylogenetic tree. The same bioinformatics analysis was performed with M2 ectodomain (M2e). The phylogenetic tree of the consensus and H5N1 M2e is shown in Fig. 1b.

B. Antigen expression and immunogenicity of H5- and M2-based DNA vaccine

The consensus sequences of HA and M2e are individually constructed into appropriate vectors as DNA vaccine candidates. Those candidates could induce high antigen expression in cultured human epithelial kidney 293T cells. We then immunized BALB/c mice with two injections of 30 μg DNA at a 3-week interval and monitored the immunogenicity by serum antibody titer. The endpoint

antibody titer after two injections is >1:10000 for HA-based vaccine while 1:100 for M2e-based DNA vaccine.

C. Virus Challenge of HA-immunized mice

To assess the efficacy of the H5-based DNA vaccine against lethal infection by Vietnam reassortant (NIBRG-14), vaccinated mice were challenged with 50 LD50 of live virus intranasally 14 days after the 2nd DNA plasmid injection. The immunization induced complete protection against lethal viral challenge measured by survival (Fig. 2a) as well as the extent of body weight loss (Fig. 2b) compared with controls.

Most recently, we have succeeded in generating psueudotyped virus like particles with envelops containing H5 from 6 different H5N1 strains, including VN 1104, VN1203, HK2003, Qing Hai, Indonesia and Tamsui. Notably, the infectivity of all of these 6 pseudotyped viruses on MDCK cells were neutralized by antisera obtained from mice 2 weeks after immunization with 30μg DNA plasmid containing consensus HA. The neutralization titers ranges from 100~500 dilution whereas the infectivity of VSV-G pseudotyped virus was not suppressed, showing the specificity of the antisera (Fig.3). This finding suggest that our H5 DNA vaccine may confer broad spectrum of protection against H5N1 of different clades, which is a clear advantage over the convential whole virus vaccine with restricted range of protective immunity.

D. Design of novel glycolipid analogs of α-GalCer

Dr. Wong's group has designed 16 structure-based synthetic α-GalCer analogs

according to computer modeling of docking of terminal phenyl group in the acyl chain and phytosphinogine tail of α-GalCer into A' pocket of CD1d molecule. They were divided into four groups including glycolipids of bacterial origin (I), modification with sulfonation (II), phenol-alkyl chain analogs (III), and phytospingosine truncated analogs (IV). His group had synthesized milligram quantities of each novel analogs and α-GalCer to facilitate studies of their immune modulating activities by Dr. Yu's group.

E. Evaluation of immune-modulating activities of novel glycolipid analogs of α-GalCer

In vitro studies showed that glycolipids were presented by CD1d molecule on dendritic cells to stimulate NKT cell expansion and cytokine/chemokine production. We found that glycolipids containing an aromatic ring in their acyl tail (group III) or spingosine tail (group IV) were significantly more effective than α-GalCer (C1) in inducing Th1 cytokines/chemokines (Fig. 4), TCR activation and human NKT expansion, esp. C11, C13, and C16.

In vivo studies of glycolipids in mice revealed that among three routes of administration, the order of potency in cyokine induction was I.V.>I.M.>S.C.. In addition, Taken together, these findings indicate that novel α-GalCer analogs can be designed to favor Th1 biasd immunity with greater immune enhancing activities than α-GalCer.

F. Adjuvant effect of glycolipids on protein vaccine

While DNA vaccine for H5N1 is being developed, we tested the hypothesis that α-GalCer and its newly synthesized analogs may enhance immune responses to

existing protein based vaccine such as tetanus toxoid. Mice were vaccinated tetanus toxoid (TT) with/without glycolipids on day 0 and day 28. The serum was harvested weekly for determination of anti-TT-specific antibodies. As shown in Fig. 5, production of anti-TT-specific IgG antibody was enhanced by C1 and C11. Although the kinetics of anti-TT production was similar to that induced by conventional adjuvant alum, C1 elicited significantly greater antibody production than alum. When the conventional tetanus toxoid with alum was combined with C1 or C11, the antibody response was further augmented to ~2 fold of conventional vaccine. These findings indicate that C1 and C11 had adjuvant effect which is synergistic with alum to further augment immune responses.

It is noteworthy that the adjuvant effects of glycolipids were remarkably durable. Twenty weeks after the second immunization, a booster dose of TT alone (without alum or glycolipids) in mice led to a rapid rise of anti-TT antibody 1 week later. The level of antibody in mice treated with C1 or C11 was twice as high as those given tetanus toxoid with alum, and more than 25 fold higher than those injected with tetanus toxoid only (Fig. 6). These findings suggested that C1 or C11 have effects on the memory T and B cells leading to an augmented booster immune response.

G. Adjuvant effects of glycolipids for peptide vaccine

While we are developing DNA vaccine for M2, we evaluated the adjuvant effects on peptide containing extracellular domain of M2 protein of H1N1 virus strain was synthesized. The amino acid sequences were

MSLLTEVETPIRNEWGCRCN. Female BALB/c mice were vaccinated with 5 or 45 μg of M2e peptide +/- glycolipids (C1, C9, C11, C14, C17) on week 0, 3, and 6. As shown in Fig. 7, 2 weeks after the 3rd immunization, the M2e peptide alone induced anti-M2e-specific IgG titer of 1.8×10^5 and 5.4×10^5 for 5 and 45 μg antigen dosage, respectively. When combined with glycolipids, 10~30 fold higher anti-M2 antibody titers were obtained. Among the 5 glycolipids tested, C11 had the best adjuvant effect which was equivalent to complete freund adjuvant but 3 fold higher titer than C1. The remaining glycolipids (C9,14 and 17) were equivalent to C1. These findings suggest that α-GalCer and its analogs have strong adjuvant activities for peptide antigens with those containing aromatic ring in the acyl tail such as C11 being most potent.

H. Future work

With the successful preliminary work, we will facilitate vaccine development by:

(1) *Monitoring the cross protection induced by HA DNA vaccine.* With no other H5N1 viruses currently available for challenge test in Taiwan, we will approach this aspect by neutralization test using pseudotyped virus. The pseudotyped virus will be produced using HIV backbone in which individual HA of different strains are used instead of the retroviral envelop proteins. Meanwhile, we will try to get other viruses for the challenge experiments. As for the neutralization test using the virus, we may collaborate with other institutes, such as CDC, to monitor the cross protection activity of the antibodies.

(2) *Determining the lowest DNA vaccine dose with and without glycolipids*. The

DNA vaccine dose that we tested is 30 μg. From our experience, we should be able to reach the same degree of protection with less amount of DNA. We are conducting a DNA dose titration in vivo and searching for appropriate glycolipid(s) in combination to decrease the DNA dose that can mediate protection against a lethal virus challenge.

(3) *Designing bivalent HA/M2 vaccine and evaluating their immunogenicity and protection.* The original of M2e DNA vaccine which encodes only one copy of M2e domain failed to induce reasonable serum antibody titer. We will optimize M2e-based vaccine by incorporating multiple copies of M2e and other carrier proteins to increase the immunogenicity. We may combine both HA and M2e-based vaccine in one injection and evaluate the immunogenicity and the protection against H5N1 virus challenge.

二、DNA 疫苗發酵量產製程開發及放大工作

A. 高產率菌株之篩選

本研究選擇大腸桿菌 DH5α 及 DH10B 與質體 pADVAX I、pADVAX II 及 pVAXHA 做配對轉殖試驗，純化得到的質體 DNA 經過不同組合的酵素限制酶鑑定確認質體圖譜大小正確，所得到 6 株轉殖菌株分別在 1 公升錐形瓶規模進行震盪培養，利用 QIAGEN Plasmid Mega Kits 純化 plasmid DNA，再以單一及兩種酵素限制酶予以確認，並以分光光度計 $OD_{260/280}$ 分析其產量，藉由菌體溼重與純化得到的 plasmid DNA yield 正確算出該轉殖

株的產率。

由實驗結果顯示 ADVAX I 及 ADVAX II 不同質體在不同的宿主細胞其細胞之 OD_{600} 值及質體產率有明顯差異，在相同的宿主細胞則差異較小。而 VAXHA plasmid 在不同的宿主細胞其細胞之 OD_{600} 值及質體產率則明顯的相差 0.5 倍。因此醱酵部分擬先採用質體產率較高的 DH10B 之轉殖菌株作為初步製程開發的試驗菌株。

B. 20 公升規模醱酵製程

a. ADVAX

i. *E.coli* DH10B/ADVAX Ⅰ：

在 20 公升級之醱酵槽中，此菌株共操作了九個批次，主要探討在不同溫度及不同 Feeding rate 下，對 Plasmid DNA 產量之影響，各批次之醱酵結果分別列於 Table 1，由 Fig. 8 GRC-95002 的生長驅勢可知 OD 雖然隨著醱酵時間緩步上升，但 Specific productivity 卻在 23 hr 下降，顯示有些養分並未被充分利用在生產質體與菌體上，再經過一系列的實驗，發現在 32℃培養時能獲得較高的 OD 值 (GRC-95008)，而 35℃ (GRC-95004) 及 36℃ (GRC-95002) 的培養能獲得較高的 Specific productivity，所以擬定生產策略為先提升 OD 到達一定程度後再提升 Specific productivity，並將此一概念應用於 GRC-95009，其結果顯示 OD 值上升至 69.38，Specific productivity 雖然只到 1.33 mg DNA / g 濕菌重，這可能是因醱酵時

由於 Feeding Medium 不足所致。

ii. *E.coli* DH10B/ADVAXⅡ：

相同的方式應用到 ADVAXⅡ，亦得到類似效果 (Table 2)：

GRC-95052 (36℃)；GRC-95055 (32→36℃)。

iii. VAXHA

a). *E.coli* DH10B

Plasmid VAXHA 經過 Transferred 到 *E.coli* DH10B 後，並經過篩選後，選擇產量較高之菌株為生產菌株，並在 20-L 醱酵槽中測試其生產條件，其結果列於 Table 3，在 36℃培養下 (GRC-95101) 發覺其生長狀況類似 ADVAXⅠ和 ADVAXⅡ，Specific productivity 在醱酵末期也有下降之趨勢，檢測培養 23 hr 後醱酵液殘存之 glucose 的濃度為 0.172g/L，顯示細菌生長與代謝 glucose 的能力均下降，因此應用 GRC-95009 之醱酵模式測試 (GRC-95103)，並在 17.5 hr 增加一個饋料控制點，結果 OD 值由 25~30 提升到 52，同時 Specific productivity (23 hr) 也提升到 2.14mg/g cell wet wt. (資料未列出)，但延長培養時間到 25 hr 時，OD 與 Specific productivity 反而下降而 glucose 在醱酵液殘存的濃度為 5.12g/L，表示此時之饋料速度可能有問題需加以修正；故可能增加饋料點或能提升 Specific productivity，降低饋料速度有助提升 OD，因此在 GRC-95106 綜合二種假設，最高的 OD 值及 Specific productivity 分別為 68.5 (23 hr)與 2.47 mg/g cell

wet wt. (18 hr) (資料未列出)，但在收槽時 (27 hr) OD 下降到 66.5、Specific productivity 也下降到 1.89 mg/g cell wet wt.，這似乎顯示 17 hr 的饋料速度可能還有有調整的空間，需再進一步測試才能確認。

b). *E.coli* DH5α

Plasmid VAXHA 經轉殖到 *E.coli* DH10B 及 *E.coli* DH5α，前者已經大略測過其醱酵條件，但在實驗中發現醱酵末期會產生一個新的 Plasmid DNA 其大小約為 3Kb 左右，與 *E.coli* DH5α 生產的 Plasmid DNA 在 Agarose Gel 上的位置類似，因此為了取得大量 Plasmid DNA (由 DH5α 生產) 並同時測試醱酵條件，依目前之結果 (GRC-95151 及 GRC-95154；Table 4) 使用 glycerol 取代 glucose 作為碳源可以提高產率。

C. 細胞及 clear lysate 回收及清洗操作

利用掃流過濾設備進行含 plasmind DNA 細胞的回收及清洗。左高的剪應力(shear force) 環境下 Chromosomal DNA 非常容易斷裂而造成純化時之困擾。因此操作壓力條件須控制以避免的 fouling 形成，而影響濾速。菌體 harvest 後首先在 4℃下進行濃縮以降低工作體積，然後利用 Diafiltration 方法以利下一步進行。若是利用掃流過濾進行 clear lysate 的濃縮及透析，雖然使用不同的薄膜，但操作時所需注意事項，則是相

同的。

D. 離心機對 clear lysate 回收及 plasmid DNA isoform 的影響

要選擇適當的離心機，首先應了解要分離的進料泥漿或懸濁液的特性。其次要了解對分離操作之目的及要求，如分離液的澄清度，濾餅或脫液後固體粒子的含液量及純度等。

本研究係利用連續式碟式離心機 (Disc type bowl)。原理則是利用環狀活門啟、閉排渣口進行間歇排渣，又稱自動排渣碟式離心機。這種離心機最大處理量可達 400-500 L / hr，適合於未來 50~100 公升醱酵規模的 clear lysate 回收操作。實驗結果顯示 clear lysate 回收率為 85 %以上，在操作時因進料及轉鼓轉動時產生之角速度所造成之剪應力皆不會對 plasmid DNA 之 supercoiled form 有所影響。

E. Plasmid DNA 初步純化之製程

初期，研究的破菌樣品先以 DH10B/pADVAX I 饋料批次醱酵 (GRC-95009)樣品做測試。以大約 1132g 濕菌破菌後進行初步純化，plasmid DNA 的回收率及宿主蛋白質的去除率分別為 47.25%及 80%以上。第二次測試則以 DH10B/p ADVAX II 饋料批次醱酵 (GRC-95055) 為樣品。大約有 1318g 濕菌進行破菌，plasmid DNA 的回收率為 58.67%，

稍有提高;但宿主蛋白質的去除率變化則不明顯。雖然兩次的 plasmid DNA 回收量都在 1500 mg 以上,但距離目標 65%以上回收率仍有很大的改進空間。

中期，實驗的菌株改為 DH10B/pVAXHA，同上述破菌、分離、沉澱方式得到 plasmid DNA 的回收率。由結果看出每一單元操作之間的回收值仍不穩定，影響最後回收率。其中發現第二次膜過濾之體積與濃度有明顯關係，不論破菌量多寡，此步驟回收的體積若能大於 2200 ml 以上，對下一步 0.22 μm 過濾的回收率會有幫助。RNA 的去除量在初步純化後可以達到 96%以上，宿主蛋白質的去除率則較不穩定，大部分的蛋白質在膜過濾及鹽沉澱初步純化時就已去掉 83%以上，其餘的 17%在經過二次薄膜過濾與 0.22 μm 過濾後還可去除掉 8%,平均去除率可達到 90%以上。

至於 DH5α/pVAXHA 初步純化結果，plasmid DNA 回收率也有 72%以上，宿主 RNA 及宿主蛋白質的平均去除率分別可達到 97%及 97.5%，產量不高的原因可能與更換不同宿主細胞有關，須要在培養基及醱酵參數上作不同修正，因此以此菌株來製備 plasmid DNA 的製程也須要模擬試驗。

在初步純化過程中每一操作步驟皆取樣分析，利用 2-propanol 沉澱得到

的 purified plasmid DNA 以電泳凝膠分析，可以清楚看到每一單元操作中質體分子結構的改變與純度 (殘留的 RNA or chromosome DNA)。其中接近 marker 3kb 位置出現了一個未知物，最後決定在 GRC-95106 饋料批次醱酵期間密集取樣追蹤，經電泳凝膠分析結果確定該未知物在 E.coli 生長 13 小時後可以被目測到存在於所要純化的 plasmid DNA 內，為進一步確認，決定以單一酵素限制酶作用，結果未知物在電泳凝膠中除了原有的 plasmid linear form 以外，並未見其他 form 存在。

F. 質體 DNA 之製程純化

由於大量生產基因治療質體分子之純度需求極高，因此去除非超螺旋質體 (包括 linear from 及 open circular form) 便成為重要課題。本計劃選用 ion-exchange chromatography 將破菌後大部分非質體分子去除， 再利用疏水性膠體作為純化質體分子之基質，大量純化所需之超螺旋形式之質體分子，同時配合不同分析方法檢測其純度。

實驗之起始樣本是將已前處理過之 DNA lysate 樣本，由於分子本身電荷差異之特性，經由小量測試發現利用陰離子交換樹脂配合適當之平衡緩衝液，可將大部分之蛋白質及 RNA 分子於 flow thru fraction 即洗出，而 plasmid DNA 則需搭配更高濃度之鹽才能將其 elute 出來。由膠體電泳 (Fig. 9) 及 HPLC 純度分析 (Fig. 10) 發現，於 IEC 純化過程中，可將大

於 98%以上蛋白質及大部分 RNA 分子去除，並回收大於 80%以上之 plasmid DNA。只不過在測試不同 plasmid DNA 純化效果時，發現 ADVAX I 和 ADVAX II plasmid 的 loading capacity 比 pVAXHA 來的好 (約多 1/3)。推測原因可能是 plasmid DNA size 不同，其電荷即不同，對 IEC column 之吸附力即不同所致。由於經由 IEC column 純化後之樣本仍含有少量之非超螺旋質體，故將此樣本進行更進一步純化。

經由鹽濃度及疏水性的改變，可將不同形式之質體利用疏水性膠體分開。於膠體電泳片兩種質體之相關位置可判定位置較下方之質體形式為 supercoiled form 之 plasmid DNA。樣本利用疏水性膠體層析純化後，質體回收率約有 85%，而超螺旋質體純度則由第一步離子管柱層析純化後之 85%提高至>97%。此外，內毒素的含量也低於 0.1 eu/mg plasmid DNA，降低約 100,000 倍 (Table 5)。每一批 20 公升規模醱酵，可得純化之 plasma DNA 約 1 克。以目前 GLP 的製程，可以達到的規模如 Table 6 所示。

因此藉著 HPLC 簡易快速 plasmid DNA 及 RNA 定量方法之建立，於整個醱酵、回收及純化流程中監控 plasmid DNA 及 RNA 雜質之回收/去除率。當醱酵回收之 cell 經 alkaline lysis、膜過濾及鹽沉澱等前處理後，在純化製程開發過程中，將會更努力地使產物之回收率、純度及穩定度皆

更提高。

G. 醣脂量化合成

a. 依計畫書所提之合成途徑，以 1 克的起始物 36 進行醣基化受體部份，重覆進行數批次的測試，已成功製備 500 mg 醣基化受體 40 (Fig. 11)。

b. 合成中間體 38 時，以 LHMDS 取代 n-BuLi 可將產率由 30%提升至 60%。

c. 建立管柱色層分析方法分離 E 與 Z- alkene(38)。

d. 使用 3 倍劑量的 benzoyl chloride 可增加醣基化受體 40 的產率。

e. 醣基化予體部份 41，由於反應的產率太低，反應條件仍在改良中。

(4) 結論

1. 何大一院士的研究團隊利用先前 H5 基因的高保留性 DNA 序列，已建構下列 H5N1 禽流感病毒的重組 H5 DNA：

   A/Hong Kong/213/03、A/Viet Nam/1203/2004、A/Viet Nam/1194/2004、A/Ck/Indonesia/5/2004、A/Bar-headed Goose/Qinghai/62/05。

   經 H5 DNA 疫苗投藥後可完全保護小鼠，體重變化與存活率 2 大指標均不受禽流感疫苗株 NIBRG-14 感染之影響。

   另也已製備 M2e 蛋白的高保留性 DNA 與一新型載體蛋白的融合基因，以增加 M2e 蛋白的表現量。

2. 翁啟惠院士的團隊持續大量合成 α-GalCer (C1)，進行免疫調節效力的動物試驗，並已設計研製 17 種 C1 的類似物。

3. 陳鈴津博士的團隊測試 17 種醣脂類似物活化自然殺手 T 細胞的效能，

發現具芳香環的醣脂類似物 (尤其是編號 C10、C11、C13、及 C16 四種類似物)，可比 α-GalCer C1 更有效促使細胞分泌 gamma 干擾素 (IFN-γ)；而各種醣脂類似物都能較 α-GalCer C1 降低白細胞介素 4 (IL-4) 的分泌。由 IFN-γ / IL-4 分泌量的比值判斷，類似物 C14、C9、C6、C10、C11、C15、C16、C7、C12、及 C13 所引發的免疫反應較類似 Th1 型；類似物 C1、C3、C4、C5、C8、及 C17 所引發的免疫反應較類似 Th2 型。

4. 在小鼠活體試驗中，以醣脂激發激發細胞激素與趨化素表現的強度高低，投藥途徑依序為：靜脈注射 > 肌肉注射 > 皮下注射。
5. 本研究將破菌規模由小量 (180 g 濕菌) 規模放大到 800 g 濕菌 (半槽) 和 1400 g 濕菌 (整槽)，結果顯示 plasmid DNA 的回收率和 supercoiled form plasmid 的比例皆可達到工業生產的水準，同時回收純化的過程中, 能有效地去除宿主 RNA 和宿主蛋白質，對後續 plasmid DNA 的管柱層析純化將有所助益。
6. 最適的破菌菌體濃度為 100 g/l (每克濕菌溶於 10 ml 的 Solution Ⅰ溶液中)，Solution Ⅰ 、Solution Ⅱ與 SolutionⅢ以等比例進行菌體懸浮、破菌與中和。
7. 在製程放大下，回收破菌完之 clear lysate 是一個很重要的單元操作，其會影響整個製程的操作體積。因此，本研究以 SA-1 連續式離心機進行 clear lysate 的回收，結果顯示回收效果良好，回收率為 65 %以上，且不會對 plasmid DNA isoform 比例造成不利的影響。
8. 利用掃流過濾膜操作能有效將 plasmid DNA 進行回收與純化，本研究未來將把掃流過濾膜技術應用在更大規模的 plasmid DNA 生產與純化製程中。

9. 已能運用 ADVAX Ⅰ/Ⅱ DNA 疫苗建立 20 公升醱酵、回收及純化製程技術。在 20 公升醱酵規模的操作下，從菌體回收、破菌、離心、薄膜過濾、沈澱等實驗流程僅需 15 個小時便可完成，顯示本製程相當具有競爭力。未來將繼續將製程規模擴大到 50~100 公升的醱酵規模。
10. Plasmid DNA 的回收率及 supercoiled form 的比例平均可達到 65 %及 90.6 %。宿主 RNA 及宿主蛋白質的去除率則平均分別達到 96 %及 90%。
11. 利用離子交換及疏水性管柱層析搭配 UF/DF 操作等方式，我們成功地將 supercoiled form 質體分子純化出來，此純化製程回收率達 40%以上，且純化產物 supercoiled form plasmid DNA 之純度可達 99%以上。最終產物中之殘留蛋白質含量<0.005%，而 OD_{260}/OD_{280} 值亦達 1.90 以上。

(5) 建議

1. 如需詳細測試我們所製作出之疫苗可保護動物免於何種流感病毒株系之感染，則需多種 H5N1 病毒株以便進行測試。但目前除越南疫苗株外，並無其他 H5N1 病毒株可用來進行攻毒或中和試驗。我們正嘗試經由何大一院士在國外的合作關係，引進其他 H5N1 病毒株，如有其他單位可互相配合，則可加速此一合作關係的建立。或有其他方式同步進行引進別種 H5N1 病毒株。此外，我們亦期盼國內能簡化輸入 H5N1 病毒株的作業流程，以利在有限的期程內順利進行計畫。
2. 以中研院基因體中心所選殖的 pVAXHA 禽流感 DNA 疫苗，運用所建立之製程技術，進行生產製程開發。
3. 調查國內醱酵 GMP 藥廠之能量。國光生技具有 75 公升醱酵設施及回收純化設施，惟尚未申請 GMP，產能亦有限。中化合成生技具 100~1,000 公升醱酵設施，但下游回收、純化設施仍然缺乏，有待建立。生技中心

之 CGMP 生技藥品先導工廠的純化設施可配合上游 GMP 醱酵及回收生產，純化 3~15 g DNA 疫苗，若增設大型管柱，則可純化 40g DNA 疫苗/批次。

4. 若未來 DNA 疫苗發展順利，防疫上有量產需求，則需儘早規劃量廠工廠。可在委外生產 (如德國 Boehringer Ingelheim 公司) 或國內自行設廠當中做一考慮。
5. 執行計畫每月需繳交進度報告，請考慮是否有其必要性。建議可改為每季繳交一次即可，如此可節省一些時間，讓研究人員能全心投入研究，貴局亦可掌握研究進度。

(6)計畫重要研究成果及具體建議

1.計畫之新發現或新發明

a. 根據流感病毒血凝素 HA 基因及病毒基質 M2 蛋白基因設計抗 H5N1 流感病毒的 2 種 DNA 疫苗，二者在體外實驗上均可誘發免疫保護效能。
b. 醣脂類似物 C11、C13 及 C16 在激發 Th1 細胞激素/趨化素表現、T 細胞受體活化、及人類自然殺手 T 細胞增殖、刺激細胞分泌 gamma 干擾素等效力，都較原型 C1 醣脂大為提高。
c. 建立克級 DNA 疫苗製程技術，未來可逐步放大，以利產業生產。

2.計畫對民眾具教育宣導之成果

a. 對禽流感防治之新型 DNA 疫苗，國內能掌握關鍵製程技術。

3.計畫對醫藥衛生政策之具體建議

a. 建議國內能簡化輸入 H5N1 病毒株的作業流程，以利在有限的期程內順利進行計畫。

b. 建議衛生署與現行具醱酵設施之 GMP 藥廠討論如何建立量產 DNA 疫苗之 GMP 設施 (需由目前所建立之技術放大 100~1,000 倍，以便能生產 100 g ~ 1,000 g /批次) 以因應未來防疫所需疫苗之需求。

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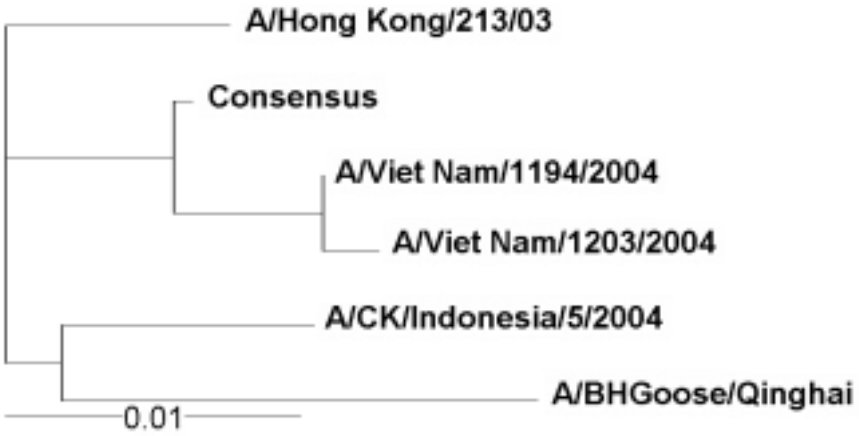
(8) 圖、表

(a) HA



(b) M2 ectodomain (M2e)

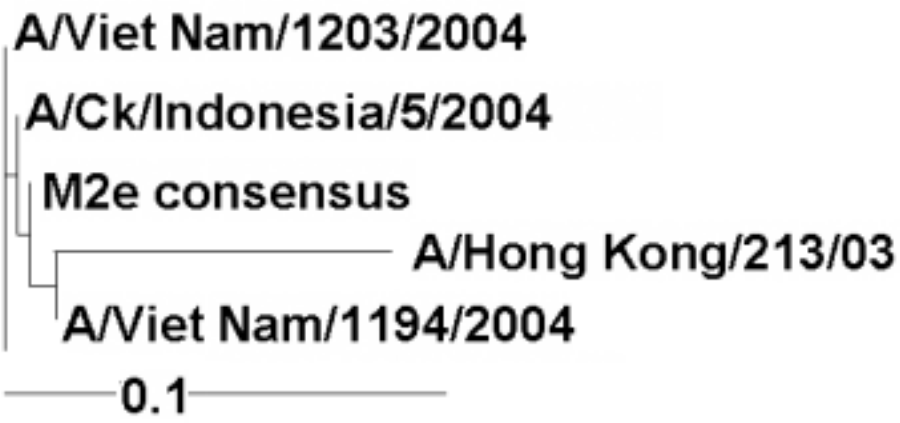
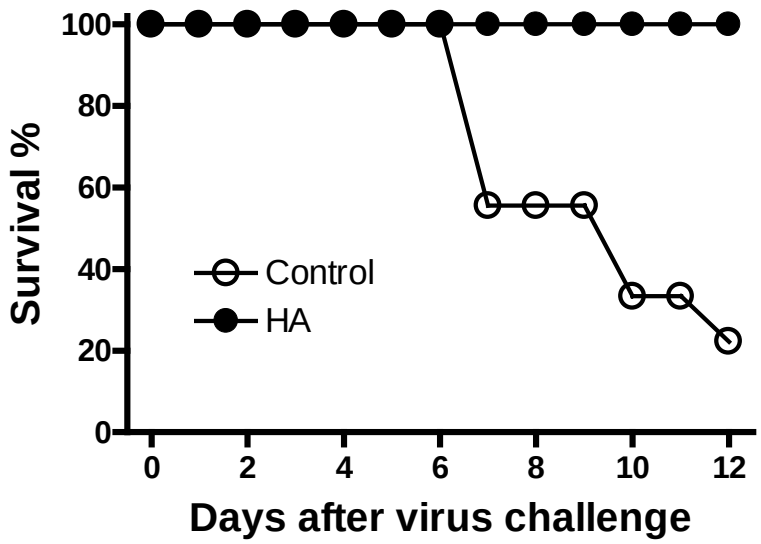


Fig. 1. (a) The phylogenetic analysis of consensus HA versus HA of WHO H5N1 vaccine strains. (b) The phylogenetic analysis of consensus M2e versus M2e of WHO H5N1 vaccine strains.

(a)



(b)

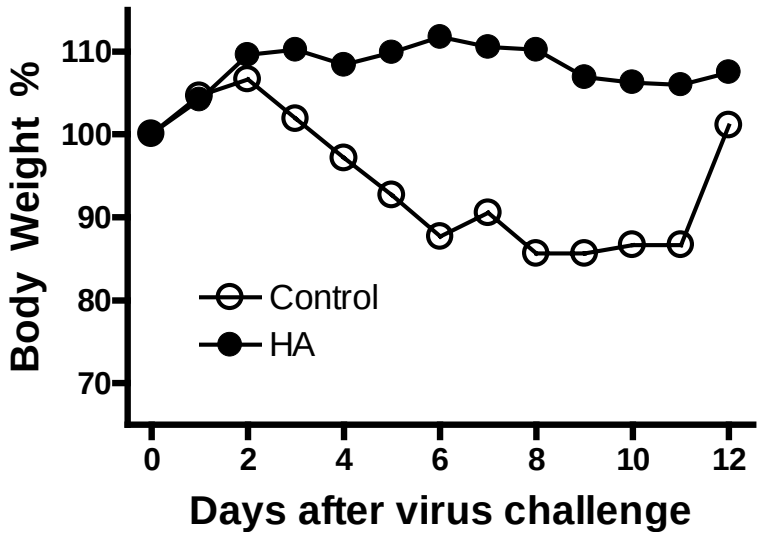


Fig. 2. Immune protection against lethal challenge of the NIBRG-14 virus. Mice were immunized (n=9 in control group, n=8 in HA group) with the consensus HA plasmid (HA) or control plasmid expression vector (Control) followed with challenge of 50 LD50 live virus. The survival (a) and the weight loss (b) were evaluated.

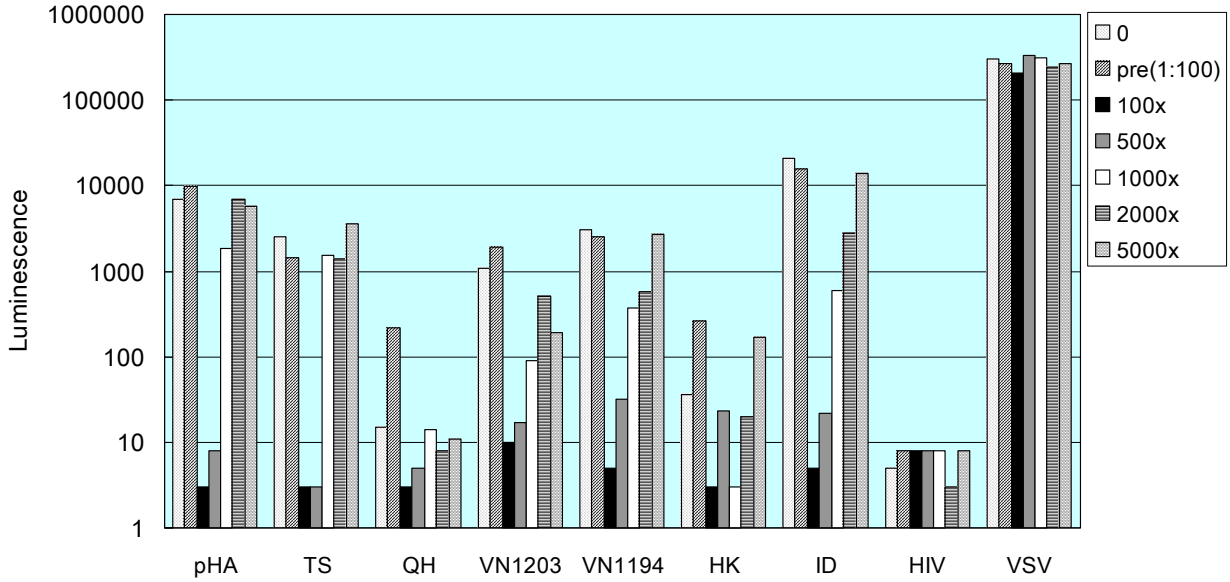


Fig. 3. Neutralization of infectivity of pseudotyped H5N1 viruses on MDCK cells by immune serum obtained 2 weeks from mice 2 weeks after 2nd immunization with H5 DNA vaccine at 30 μg/mouse.

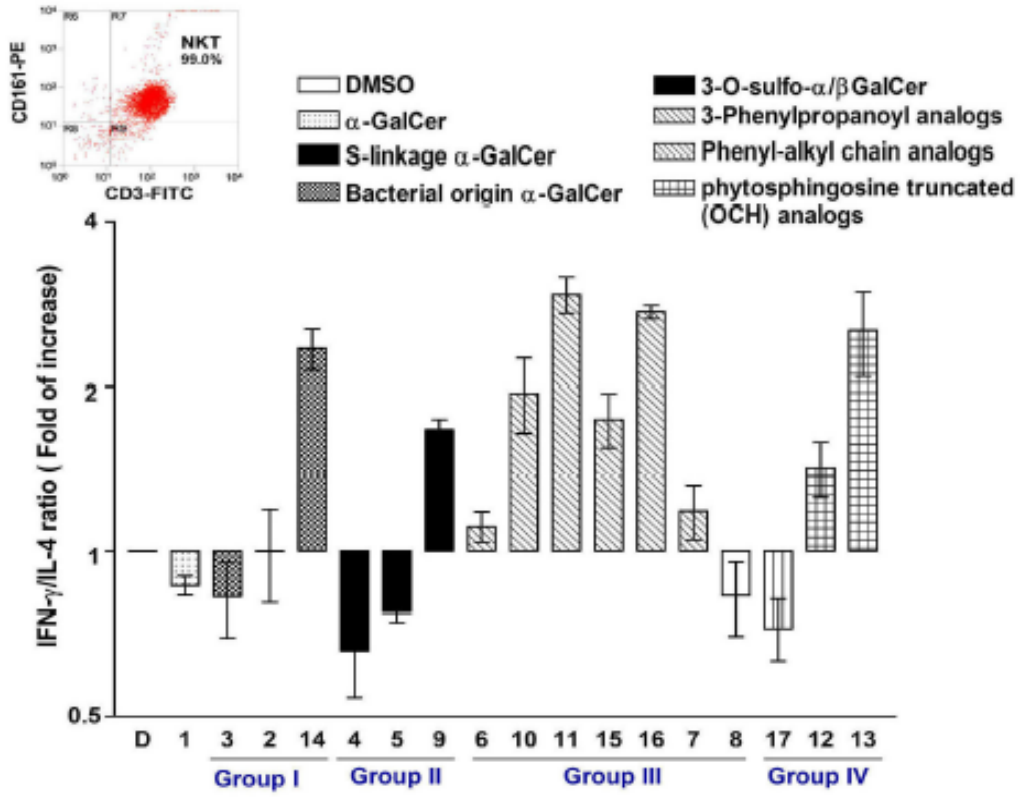


Fig. 4. Th1/Th2 cytokine production by human NKT cells in response to novel glycolipids.

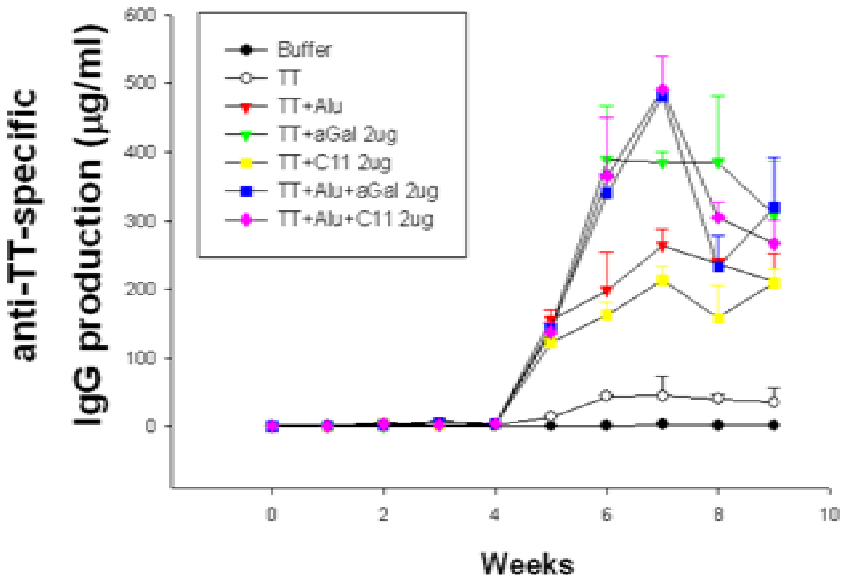


Fig. 5. Adjuvant effects of glycolipids on antibody Response to tetanus toxoid.

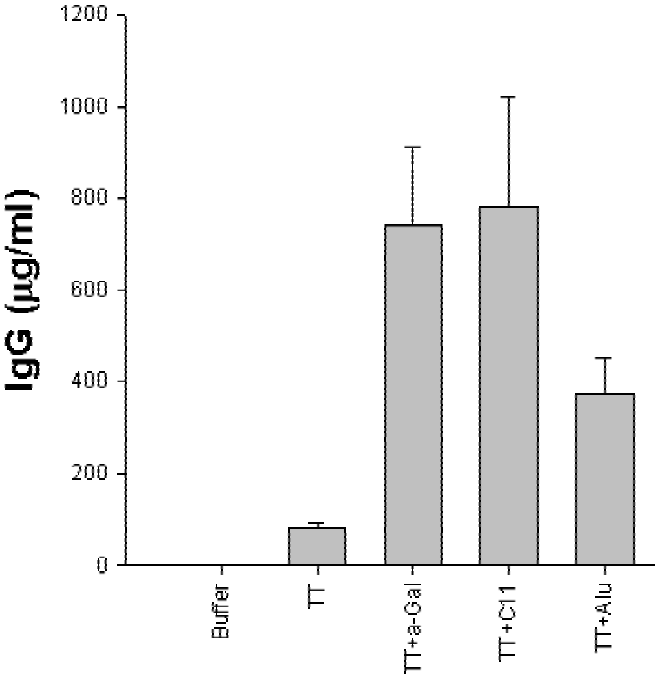


Fig 6. Effects of glycolipids on delayed antigen boost (20 weeks after 2nd vaccination).

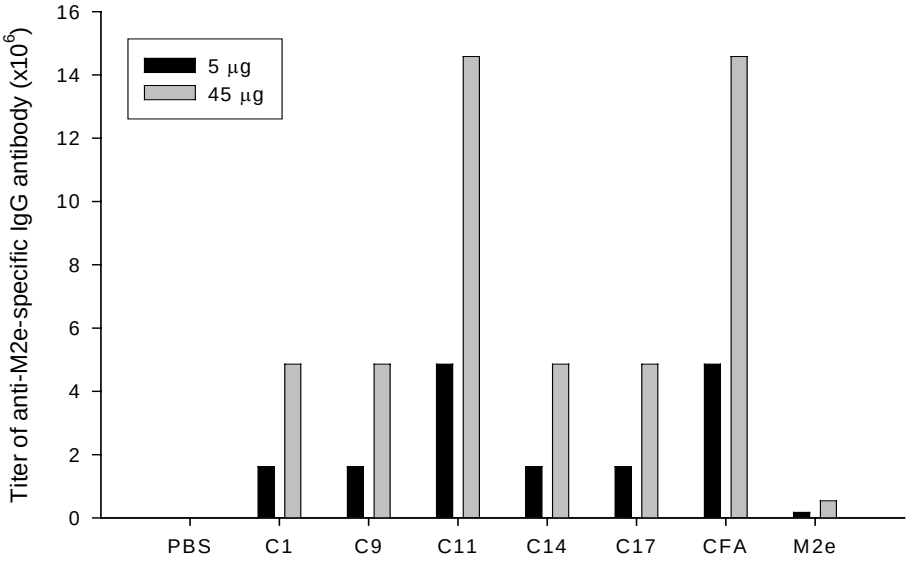


Fig 7. Adjuvant effects of glycolipids on M2e peptide vaccine.

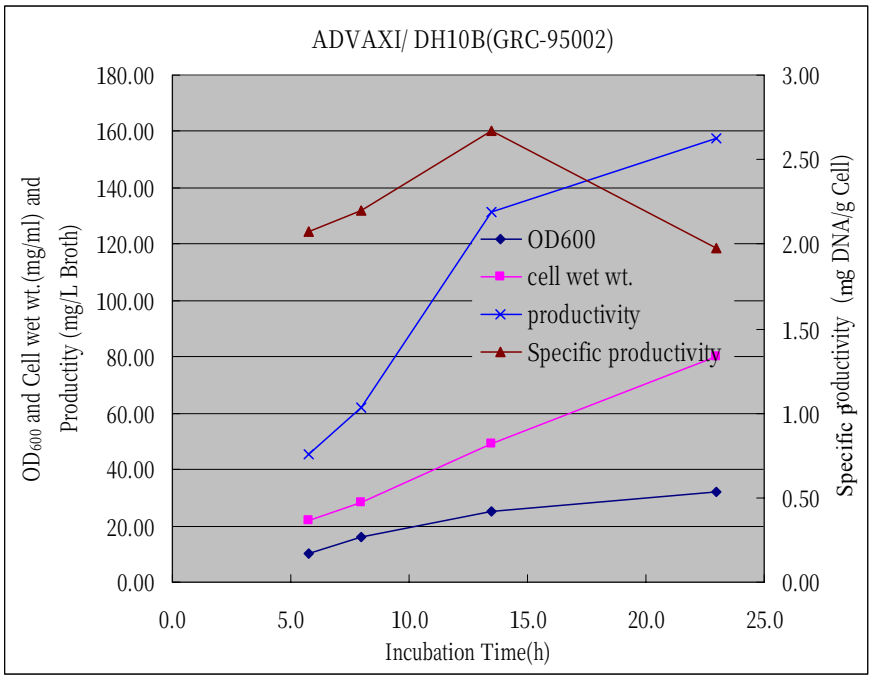
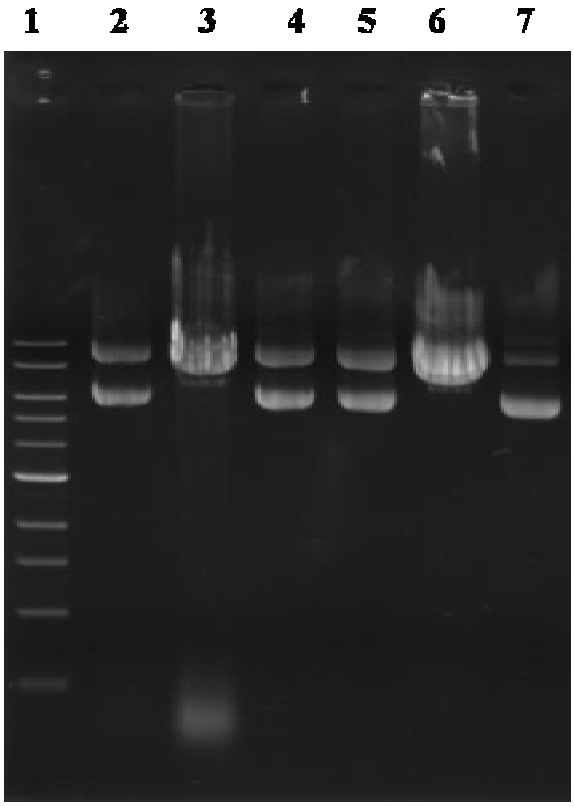


Fig. 8. Fermentation Result of GRC-95002.



Lane1: marker

Lane2: 0.2 u filtrate

Lane3: IEC F.T.

Lane4: IEC elute

Lane5: UF/DF of IEC in 10xTE

Lane6: HIC F.T.

Lane7: HIC elute

Fig. 9. Agarose electrophoresis analysis of VAXHA.

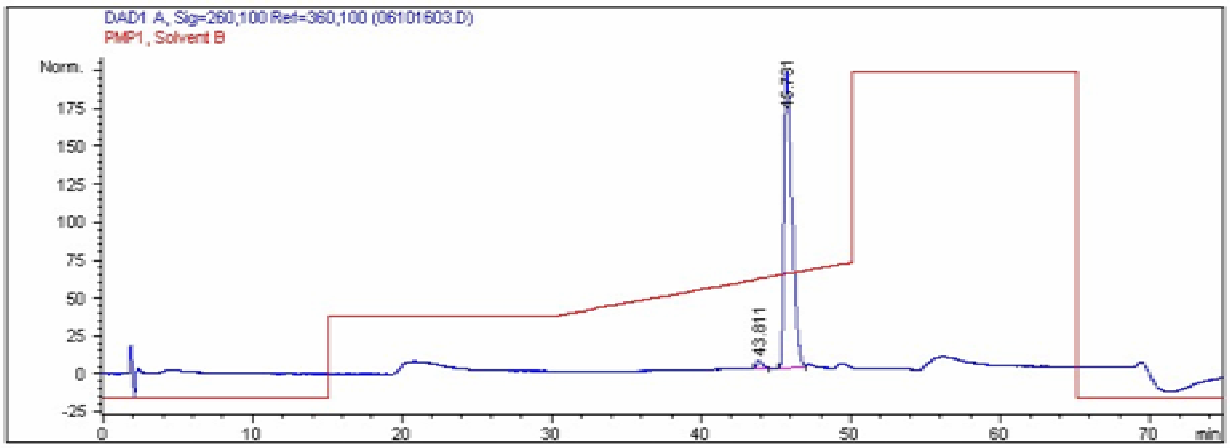


Fig. 10. HPLC analysis of VAXHA after HIC step.

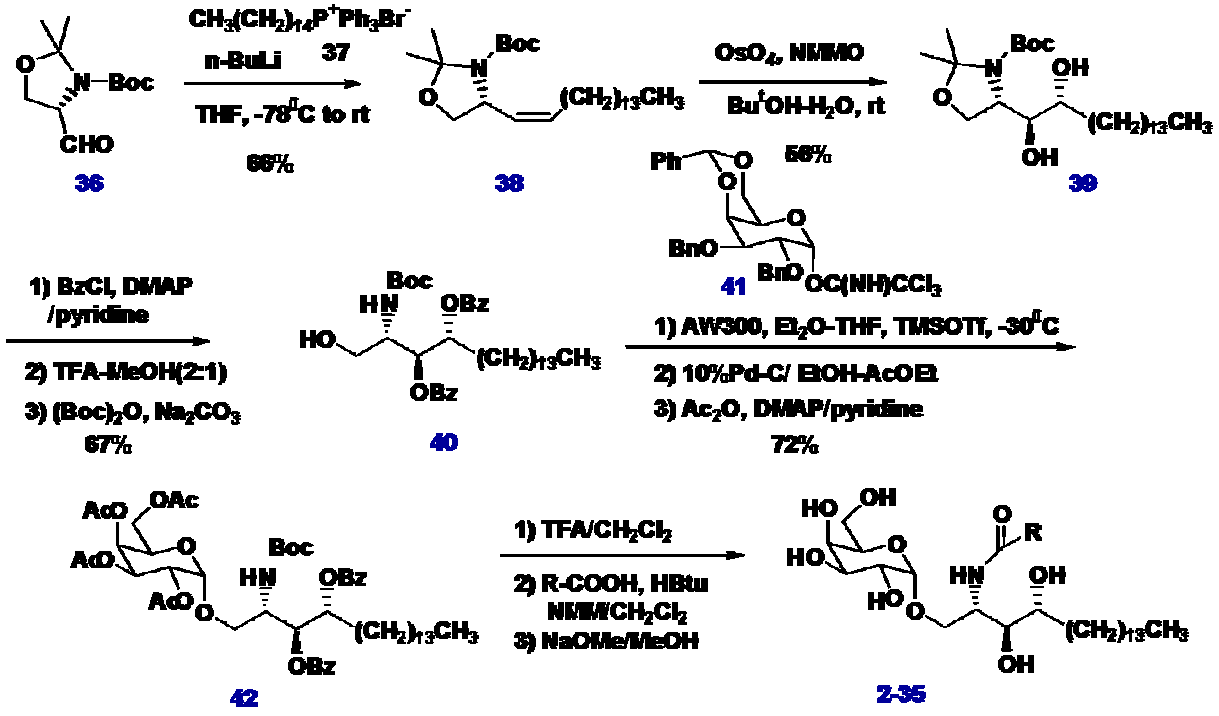


Fig. 11. Synthesis of α-GalCer analog.

Table 1. 20-L Fermentation result of *E.coli* DH10B/ADVAX I.

Item	Duration (hrs)	Temp.	OD_{600}	cell wet wt. (mg/ml)	Specific productivity (mg DNA / g cell wet wt.)	productivity (mg DNA /L Broth)
GRC-95002	23	36.2	32	79.87	1.97	157.52
GRC-95004	27	35.9	26.8	74.96	1.94	145.26
GRC-95008	23	32.1	43.56	98.1	1.36	133.65
GRC-95009	23.5	32→36	69.38	121.83	1.33	161.49

Table 2. 20-L Fermentation result of *E.coli* DH10B/ADVAX II.

Item	Duration (hrs)	Temp.	OD_{600}	cell wet wt. (mg/ml)	Specific productivity (mg DNA / g cell wet wt.)	productivity (mg DNA /L Broth)
GRC-95052	23	36.1	40.8	90.115	1.48	132.99
GRC-95055	23.5	32→36.3	65.5	104.63	1.35	141.15

Table 3. 20-L Fermentation result of *E.coli* DH10B/pVAXHA.

Item	Duration (hrs)	Temp.	OD_{600}	cell wet wt. (mg/ml)	Specific productivity (mg DNA / g cell wet wt.)	productivity (mg DNA /L Broth)
GRC-95101	23	35.7	30.8	68.11	1.80	122.91
GRC-95103	25	32→36.2	52.5	118.7	1.97	234.02
GRC-95106	27	32→35.9	66.5	149.6	1.89	282.38

Table 4. 20-L Fermentation result of *E.coli* DH5α/pVAXHA.

Item	Duration (hrs)	Temp.	OD_{600}	cell wet wt. (mg/ml)	Specific productivity (mg DNA / g cell wet wt.)	productivity (mg DNA /L Broth)
GRC-95151	28	35.9	33.6	89	0.51	45.5
GRC-95154	27	36.2	48.8	106.8	0.82	87.4

Table 5. Data of VAXHA plasmid.

Information	Vol. (ml)	HPLC-Conc. (mg/ml)	HPLC-Amount (mg)	SC (%)	Recovery (%)	Protein-Conc. (μg/ml) BCA	Protein / DNA	OD_{260} / OD_{280}
0.22 μm filtration	2400	0.454	1089.6	91.2	100.0	2.88	0.634%	1.87
IEC elute	4665	0.197	919.1	93.5	84.3	0.05	0.026%	1.86
HIC elute	3474	0.202	701.75	100	64.4	0.01	0.005%	1.89

Table 6. QC item for plasmid DNA (GLP grade).

Test	Specification	Result of VAXHA
1. Appearance	Clear, colorless solution	conform
2. Identity of the Plasmid	Restriction fragment size conforms	conform
3. DNA homogeneity	Supercoiled isoform >95%	SC form >99% by HPLC
4. DNA concentration	Expressed in mg/ml	0.2 mg/ml
5. A_{260}/A_{280}	1.80-2.0	1.90
6. Scan 220-320 nm	Conforms (peak at 260 nm)	conform
7. Genomic DNA	Undetectable < 5%	In process of analysis by real time PCR
8. Total protein	< 5%	< 0.005% by BCA colorimetric assay
9. Host RNA	Not detectable by HPLC	Conform by HPLC
10. Endotoxin (LAL)	<350 EU/dose	<90 EU/mg

附件一 計畫主持人自評

計畫編號：DOH95-DC-1411

計畫名稱：研發以新型醣脂佐劑結合抗人類流感及禽流感之雙效疫苗

計畫主持人：翁啟惠　　服務單位：中央研究院基因體研究中心

聯絡地址：台北市南港區 115-29 研究院路二段 128 號　電　話：02-2789 9400

傳　　真：(02) 2789 9924　E-mail：chwong@gate.sinica.edu.tw

研究性質與期程

一、研究性質：

☑基礎研究

☑應用研究

☑技術發展

二、計畫期程：3 年 (95/1/1～97/12/31)

三、計畫經費（仟元）

第一年：22,000

第二年：33,500

第三年：34,500

計畫預計目標（OUTPUT）

第一年：融合基因的設計與構築，以及試管內表現定性

設計並合成醣脂

DNA 疫苗進行量產試驗 - 以 ADVAX plasmid 建立製程平台，再以

候選禽流感 DNA 疫苗完成製程開發及批次量產測試(20 公升規模)，並且調查國內具醱酵設施之 GMP 工廠，以配合製程放大需求。

第二年：在模式動物中進行 DNA 疫苗之免疫原性分析

測試醣脂輔助 DNA 疫苗的佐劑效能

制定候選禽流感 DNA 疫苗之標準化製程、進行 H5/M2 疫苗 50-100 公升醱酵規模製程放大試驗

第三年：在模式動物中評估疫苗對流感的預防力

評估佐劑結合疫苗之免疫反應

進行臨床前藥理安全及毒性試驗

針對選定之候選禽流感疫苗完成製程開發及標準化，完成 50~100 公升醱酵規模之 plasmid DNA 純化製程放大。

預期效益（OUTCOME）

研發有效對抗人類流感及禽流感的雙效疫苗，完成禽流感 DNA 疫苗製程開發、製定標準化製程並實施候選之疫苗放大製程建立。

A. 利用疫苗載體重組 H5 及 M2 的部分序列成為融合基因。

B. 合成數種可刺激人類及小鼠 NKT 細胞的醣脂。

C. 評估上述 DNA 疫苗與醣脂一起施用的佐劑效力。

D. 與生物技術開發中心合作，經由優良實驗室規範生產疫苗，進行臨床前藥理、毒理試驗，以便申請新藥試驗。

E. 完成 DNA 疫苗製程開發、制定標準化製程並建立 DNA 疫苗 20 – 100 公升醱酵及純化技術，以便未來可協助 GMP 藥廠建立 DNA 疫苗量產技術

自訂績效指標

■ 期刊論文

本計畫為 3 年計畫，目前為第 1 年，暫時未有期刊論文之產出；預計在計畫結束前可發表 6~10 篇相關學術研究成果在頂尖學術期刊。

■ 研討會論文

暫時無。

■ 專著

暫時無。

■ 技術報告

暫時無。

■ 技術創新

暫時無。

計畫參與人力

	第一年	第二年	第三年
博士級	14		
碩士級	16		
學士級	1		
專科	4		
其他	1		
合計	36		

計畫執行情形

■ 是否依計畫預計進度執行？

是

■ 兩次進度報告中審查委員對本計畫之建議？

另附。

■ 是否遭遇執行上之困難？

如需詳細測試我們製作出之疫苗可保護動物免於何種流感病毒株系之感染，則我們需多種 H5N1 病毒株以便進行測試。但目前除越南疫苗株外，並無其他 H5N1 病毒株可用來進行攻毒或中和試驗。我們正嘗試經由何大一院士在國外的合作關係，引進其他 H5N1 病毒株，如有其他單位可互相配合，則可加速此一合作關係的建立。或有其他方式同步進行引進別種 H5N1 病毒株。此外，我們亦期盼國內能簡化輸入 H5N1 病毒株的作業流程，以利在有限的期程內順利進行計畫。

■ 是否修改計畫？

見附件二。

■ 經費執行情形？

除人事費因經費撥付較遲，前一季幾乎沒能及時僱用研究助理，故執行率較不佳，餘額將轉入研究業務費，目前執行為 88% (計算日期：95/11/10)。

階段成果-目標達成

■ 達成什麼目標

(1) 已根據 HA 的高保留度序列或 M2 的外露區域設計重組為融合基

因，並利用 pVAX 載體表現產生 DNA 疫苗。

(2) 經 H5 DNA 疫苗投藥後可完全保護小鼠，體重變化與存活率 2 大指標均不受禽流感疫苗株 NIBRG-14 感染之影響。

(3) 已設計並合成各型醣脂類似物計 28 種。

(4) 以調節 NKT 細胞免疫作用的效力篩選各型醣脂，發現 C11、C24、及 C25 較原型 C1 醣脂具有更高的效能。

(5) 以 ADVAX DNA 疫苗建立 20 公升醱酵、回收及純化製程平台，完成製程開發及 20 公升規模批次量產測試，預估可得 1 克左右純化 DNA 疫苗。

■ 論文產出或技術創新簡述

暫時無。

特殊績效

暫時無。

階段成果-研發團隊

團隊名稱	研發以新型醣脂佐劑結合抗人類流感及禽流感之雙效疫苗	
姓名	學歷	工作內容與專長
翁啟惠	博士	領導、規劃、統籌與協調本研究計畫執行，整合研究資源與人力，負責計畫研究及行政業務之推展，並負責發展的新型醣脂作為建構疫苗的佐劑分支計畫之研究工作。
陳鈴津	博士	協助計畫主持人研究計畫執行，及計畫研究及行政業務之推展，並負責流感疫苗臨床前期研究及轉譯醫學研究工作，及追蹤臨床研究分支計畫之工作。
何大一	博士	協助計畫主持人研究計畫執行，並負責流感疫苗研發分支計畫之工作。
鄭義循	博士	協助子計畫 DNA 疫苗發酵量產製程開發及放大工作進行；負責 DNA 發酵程序開發及放大。
鄭婷仁	博士	DNA 純化程序開發及放大。

吳明基	博士	計畫協同主持人；主持及協調本計畫 DNA 疫苗醱酵量產製程開發及放大工作。
黃瑞蓮	博士	計畫協同主持人；主持及協調本計畫 DNA 疫苗醱酵量產製程開發及放大工作。
紀威光	博士	研究人員;協助本計畫 DNA 疫苗醱酵量產製程開發及放大工作;DNA 醱酵程序開發及放大。
林藹寧	博士	研究人員；DNA 純化程序開發及放大。
徐悠深	博士	研究人員；DNA 疫苗分析。
郭曼娗	博士	α-GalCer 醣脂的製備規畫與推動，藥物合成與分析。
黃建智	博士	合成設計, 藥物合成。

階段成果-人才培育

無

姓名	學位	學習內容或論文題目	畢業年度
	博士研究生		
	碩士研究生		

成果自評

本計畫在翁啟惠院士、何大一院士、陳鈴津博士、以及生物技術開發中心 4 個研究團隊的通力合作之下，已順利達成預期目標：根據禽流感病毒的 HA 及 M2 基因設計並產製 DNA 疫苗，經 H5 DNA 疫苗投藥後可完全保護小鼠，不受禽流感疫苗株 NIBRG-14 感染之影響。已建立 20 公升 DNA 疫苗的醱酵、回收及純化製程平台，完成製程開發及 20 公升規模批次量產測試，預估可得 1 克左右純化 DNA 疫苗。同時也設計並合成各型醣脂類似物計 28 種，並以 NKT 細胞免疫作用的調節效力篩選出 C11、C24、及 C25 等 3 型醣脂。此研究成果將使得未來國人自製禽流感/流感疫苗能突破現有的瓶頸。

後續發展

由於本計畫已成功達到第 1 年的預期目標，我們將如計畫書所規劃，在模式動物中進行 DNA 疫苗之免疫原性分析、評估疫苗對流感的預防力、評估佐劑結合疫苗之免疫反應、

及放大 DNA 疫苗之製程，持續加速 DNA 疫苗的開發，目前正朝以下方向進行：

(1) 測試 HA DNA 疫苗對抗各種禽流感病毒株系的交叉保護作用。

(2) 測試有或無各新型醣脂結合 DNA 疫苗之最低劑量。

(3) 設計 HA/M2 雙效疫苗，並評估其免疫原性及預防感染之效能。

(4) 將 20 公升醱酵規模擴大到 150 公升。

附件二 95/7/26 疾管局「流感疫苗研發計畫第二期進度報告」會議建議事項回覆

A. 綜合建議

一. 國內發生禽流感疫情時，各計畫主持人應完全配合本局防疫措施，即時提供人力與物力資源，供本局調配運作。

本中心將全力配合。

B. 個別計畫建議

1. DNA 疫苗的免疫效果需加速測試。

H5 DNA 疫苗的免疫效果已進行測試。以不同劑量施打小鼠，可誘發小鼠體內產生抗 H5 抗體，其效價並且呈現劑量依隨效應。

2. 評估 DNA 疫苗在小鼠引發的 HI titer，其檢測方法如 HI 或 NT assay 可尋求其他計畫單位支援。

在 HI 或 NT assay 部分 已尋求疾病管制局支援。

3. 所生產之醣脂體是否可應用於 whole virus particle，宜與國衛院討論，以幫助緊急疫苗生產。

本中心已製備 2 種醣脂 (α-GalCer 及 C11) 各 1 mg，將可提供國家衛生研究院莊博士，以利進行應用於 whole virus particle 佐劑的效能試驗。

4. 醣脂體之作用機轉及用於人體之安全性宜考量，應與 CDE 做進一步討論。

經由測試各種醣脂類似物活化自然殺手 T 細胞的效能，發現具芳香環的醣脂類似物 (尤其是編號 C10、C11、C13、及 C16 四種類似物)，

可比α-GalCer C1 更有效促使細胞分泌 gamma 干擾素 (IFN-γ)；而各種醣脂類似物都能較 alpha-GalCer C1 降低白細胞介素 4 (IL-4) 的分泌。由 IFN-γ / IL-4 分泌量的比值判斷，類似物 C14、C9、C6、C10、C11、C15、C16、C7、C12、及 C13 所引發的免疫反應較類似 Th1 型；類似物 C1、C3、C4、C5、C8、及 C17 所引發的免疫反應較類似 Th2 型。

已與 CDE 接觸，建立初步討論的窗口。

5. 產程及產量之最佳化可藉由電腦產程規劃研究最佳條件。

反應曲面法醱酵模式應用電腦模擬程式，對產程及產量的最佳化，應有幫助。依現今較常使用之「反應曲面法」，若在 20 公升級以下之醱酵槽使用此方法以提升生產效能，對於生產之菌株及培養基需求與產物純度之關係雖須進一步分析，但在理論上在 20 公升級之醱酵槽中應用「反應曲面法」來提升產量理論上或許可行。

6. 20 L 產 1 克之產程可否再放大使效益更佳。

生技中心目前的設備 20 公升，產程之放大需與國內業界配合進行 50~100 公升醱酵製程(96 年度計畫)。另外亦可在 20 公升產程，進一步提高產率。

7. 儘快建立或評估合作的 cGMP 工廠生產 plasmid DNA。

國內目前並無可量產 plasmid DNA 之 cGMP 工廠，若需國內建廠，則建議衛生署由政策面與國內藥廠溝通建廠之可行性。初期可委託國外具完整設施及技術之藥廠進行 (如德國 Boehringer Ingelheim 藥廠具 6,000 公升 plasmid DNA 醱酵槽及純化設施)。

附件三 95/12/06流感疫苗研發計畫95年度成果進度審查委員意見回覆

Reviewer 1

(I) General background

The proposed vaccine uses new approach to include HA and M2e antigen genes into appropriate vectors and prepare as a DNA vaccine with a novel glicolipid adjuvat. The scientific design and approaches look reasonable and solid. However, the vaccine development will meet

enormous practical difficulties and challenges:

1. *There are no licensed products of the pandemic influenza virus vaccine, DNA vaccine and glycolipid adjuvant. The licensed products are usually used as the positive control. What positive controls you will be used to compare the safety and efficacy of the proposed vaccine candidate?*

   According to numerous clinical trials of DNA based vaccine in a variety of diseases, DNA based vaccine in general is considered to be safe. The current H5N1 vaccines in clinical trials confer narrow spectrum of protection depending on the specific viral strains chosen for vaccine development. On the other hand, our vaccine design is based on the use of consensus sequences from 500 different strains of H5N1 with the hope to confer broader spectrum of protection. Thus, if our DNA vaccine for H5N1 shows efficacy in inducing neutralizing antibodies and indeed confer a broad spectrum of protection from challenges by different H5N1 viruses in animal studies, we should be able to proceed to phase I clinical trial.

2. *Avian influenza virus H7N7, H9N2 and H5N1 subtypes have been involved in human influenza illness. What percentage (%) of cross-protective immunity, the proposed avian H5N1 antigen gene will be induced against other strains?*

   This is an intriguing question but beyond the scope of our proposed study. Although we hope that our H5N1 DNA vaccine will confer cross protection for different strains of H5N1, it is unlikely to see much cross protection for H7N7 or H9N1 because of their divergence from H5N1.

3. *Many DNA vaccine candidates show high immune responses to small animals. However, they demonstrate poor protective immunity to large animals and humans. How is the effectiveness of the glycolipid adjuvant to stimulate and enhance the proposed DNA vaccine candidate in monkeys and humans?*

This is indeed true for many DNA vaccines administered intramuscularly. However, this is not the case when DNA vaccine is given by electroporation as we do. Studies conducted in non-human primate showed DNA vaccines administered by electroporation induced potent immune responses, even better than in small animals (Ichor Medical System).

4. *Aluminum salt (Alum) remains the only adjuvant in the U.S.-licensed vaccine formulation. MF59 is licensed in Europe. Many new adjuvants have been shown to be more effective than aluminum salt in enhancing antibody and cell-mediated immune response. The main reason the new adjuvants have not been licensed is that the safety issue has not been established. Some times, the animal models may not reflect the expected toxicity in humans. It takes extensive clinical trials to establish the adjuvant safety in humans. In addition, the novel glycolipid should be applied biological license as a new product that required to examine the safety in the adjuvant alone, and in the final vaccine candidate plus the adjuvant. Usually, it will take many years to reach the conclusion.*

   We agree with the importance of conducting necessary preclinical studies of DNA vaccine and glycolipid adjuvant for future clinical trial of the final product. The prototype glycolipid, alpha-galactosyl ceramide had undergone clinical trials previously, and no dose-limiting toxicity was observed over a wide range of doses (50-4,800 micro g/m^2). Thus, we expect that novel glycolipids to have similar safety profile. However, we will have to identify which of the 16 glycolipid analogs possesses the best adjuvant activity, before embarking on the preclinical toxicology/pharmacology studies. In case of urgent need, one strategy is to use alpha-galactosyl ceramide as adjuvant for clinical trial, if proven to enhance immune responses to DNA vaccines.

5. *How soon Dr. Wong's group can finish the pre-clinical animal safety studies and clinical trials on glicolipid adjuvant to obtain sufficient safety date for license application? How soon the CDE and Dept of Pharmaceutical Affairs can review and approve the license of the new adjuvant product?*

   As mentioned above, once we identify the glycolipids of choice, we will proceed with preclinical animal safety studies. Licensure of the new adjuvant product will require many clinical trials which are beyond the scope of this proposal.

(II) Selection and design of H5- and M2e- based DNA vaccine

Selection and design of the H5N1 and M2e protein antigens were deduced from consensus HA sequence from HA gene of 500 H5N1 virus strains and polygenetic tree of the consensus H5N1 M2e.

(a). Have the adequate selection and design of the proposed DNA vaccine been confirmed or accepted by WHO or the U.S. CDC that the selection and design of these antigens would accurately predict the characteristics of pandemic influenza virus infection in Taiwan area 6 to 12 months before the outbreak of influenza illness in humans?

There are more than 500 hemagglutinin genes of H5N1 virus available in the database. The biggest problem encountered during H5N1 vaccine development is the issue of cross-protection. In order to cover the genetic variability and thus induce cross-protection across different H5N1 strains, we deduced a consensus HA sequence from HA gene of 500 H5N1 virus strains and used this consensus sequence for vaccine development effort. Hopefully, immunity induced by such H5 DNA vaccine will confer broader scope of cross-protection. Another advantage of such strategy is the ease of modifying specific nucleotide sequences should new important mutation arise.

(b). Has Taiwan CDC maintained a system or capability to conduct the epidemiological survey in Taiwan area to accurately predict what influenza virus strains will be involved in the seasonal as well as pandemic influenza virus outbreaks? Did Taiwan CDC conduct such survey in the past?

Is the survey information of influenza infection on Asian countries and other areas of the world provided by WHO directly applicable to Taiwan situation for design and manufacture of the influenza virus vaccine?

We will defer this issue to CDC in Taiwan.

(III) Evaluation of efficacy and safety on DNA vaccine candidate

Licensure of pandemic influenza virus vaccine may be sought as a supplement to an existing Biologic License Application (BLA) or a new BLA.

(a). Have the animal studies been conducted regarding how the bivalent protein antigens or the DNA vaccine final products are as effective or even better as compared to the licensed vaccines, such as "split virus" purified protein influenza virus vaccine? The appropriate endpoints may include:

(a-1) the % of animals achieving HI antibody titer >= 1:40, and (a-2) rates of seroconversion, defined as 4-fold rise in HI antibody titer post-vaccination.

The geometric mean titer (GMT) should be included in the results.

We agree with the suggested endpoints for seroconversion rate. The potential advantages for our DNA vaccine based on consensus sequences over other vaccine strategies (whole virus, split virus purified protein, etc..) are broader scope of protection, ease of production and therefore lower cost, ease of modifying the DNA sequences if indicated by epidemiology survey.

(b). Local and systemic adverse reactions and symptoms of influenza illness should be well defined in different age groups.

We will certainly take these into consideration when we develop protocol for phase I clinical trial.

(c). All influenza vaccine products formulated with an adjuvant should be as new products. Data supporting their approval should be submitted to a new BLA.

We agree.

(IV). Concerns for immunogenicity, safety, and manufacture issues on DNA vaccine

(A). Pre-clinical animal studies should be conducted to establish immunogenicity and safety:

(1). Immunogenicity – Adequate assays should be developed to assess immunological potency in animal models, including the evaluation of antigen-specific antibody titers, seroconversion rates, activation of cytokine secreting cells, and/or measures of cell-mediated immune responses. Duration of the immune response should also be examined.

We agree entirely with the suggestions, and indeed, experiments to provide these data are ongoing.

(2). Autoimmunity – Studies should be conducted to establish that systemic autoimmuity is unlikely to result from DNA vaccination.

There are extensive experiences in human trials in US and Europe and no serious adverse events have been reported. This include several dozen phase I clinical trials of prophylactic DNA vaccines and many hundreds of normal volunteers have been vaccinated. Multi-milligram doses have been administered repeatedly to the same subjects. DNA vaccination does not induce autoimmune disease in normal animals, or accelerate the onset/severity of disease in lupus-prone animals. No systemic or organ-specific autoimmune disease has been reported in DNA-vaccinated volunteers, although CpG DNA can promote the development of organ-specific autoimmune disease when co-administered with self antigen.

(3). Tolerance -- Capacity of a DNA vaccine to induce tolerance may depend on the nature of the encoded antigen and the age at which, and frequency with which, the vaccine is administered.

There is no evidence of tolerance induction by DNA vaccines in human experiences. DNA vaccines do not tolerize adult animals. Certain DNA vaccines can induce

neonatal tolerance. Thus, DNA vaccination of children and newborns will proceed only after efficacy is established in adults and we plan to evaluate tolerance using an age-relevant pre-clinical animal model prior to use in children.

(4). Local reactogenicity and systemic toxicity. – Studies designed to assess systemic toxicity may be combined with assessment of local site reactogenicity, using the highest dose of vaccine planned for clinical use.

Based on extensive human experiences in US and Europe, local reactogenicity has been mild. In our animal studies of H5 DNA vaccine delivered by electroporation, there is no obvious local reactions. We plan to carefully monitor the local site reaction in preclinical studies as well as clinical trials.

(5). Others – Genetic toxicity for integration of plasmid DNA into host genome to induce mutagenesis and chromosomal instability, Reproductive toxicity and tumorigenecity.

Chromosomal integration is rare (<30 copies per 105 host cells) following conventional vaccine delivery. However, methods that increase plasmid uptake (electroporation, liposome encapsulation) may concomitantly increase the integration rate. Since our DNA vaccine will be delivered by electroporation, the company which provides the device is in the process of examining this issue of chromosomal integration in animal study, in collaboration with Dr. David Ho. A clinical trial of DNA vaccine delivered by electroporation is planned for July 2007. This will provide important safety information for our proposed DNA vaccine for avian influenze.

(B). Manufacturing issues:

(1). Product manufacture.

-- plasmid construction should be described in detail.

-- describe the DNA sequence of the entire plasmid present in the MCB, as well s the genotype, phenotype, source of bacterial cells, and the procedures to construct MCB and WCB.

(a) The first year's target is to establish plasmid DNA vaccine process technology platform, and we have done this using ADVAX and VAXHA plasmids with success.

(b) Detailed construction of plasmid and DNA sequence are available from GRC, documentation of host cell (DH10B, DH5a) are available and traceable to cell culture collection center.

(c) R&D cell bank been constructed (and procedure available) to allow for process development in 20 liter fermentation scale. When H5/M2 construct(s) are

finalized, we will construct MCB based on the host cell tested and procedure fine-tuned. This will likely to happen in the second year.

(2). Bulk plasmid product release testing.

-- in-process testing to ensure manufacturing consistency, product safety, and stability.

-- establish specification on bacterial host contaminants, nucleic acids, and proteins

-- pyrogen test.

-- identification and potency assay.

(a) HPLC method has been established to monitor plasmid quantity, supercoiled form/open circular form, RNA during the purification process.

(b) Agarose gel electrophoresis is also used for more qualitative analysis for in-process control.

(c) Restriction map is used to confirm the identity of the bulk plasmid produced.

(d) Host cell protein and endotoxin have been followed for in-process and final bulk testing.

(e) A260/A280 ratio, scan 220-320nm spectrogram (peak at 260 nm) are used

(f) LAL testing has been used widely and approved by regulatory agency for endotoxin detection.

(g) Pyrogen test will be conducted when CGMP clinical material production is initiated.

(h) Potency of bulk plasmid DNA will be verified by GRC.

(V). Others.

If possible, make arrangement with the reviewers in Virology Division of CBER, FDA for pre-IND meeting to discuss the scientific design, and concerned issues. What general and specific data are required for IND submission? Adequacy of the proposed clinical trial proposals and related issues.

Because the proposed DNA vaccine involves development of 3 novel biological products combined in to one final container, it is a very difficult job. CBER reviewers have unique and lots of experience in dealing with various problems. They can provide useful scientific recommendations to speed-up and improve the vaccine development project.

We appreciate the suggestions and plan to do so in the next 6 to 12 months, depending on the results of our DNA vaccine +/- glycolipids in animal studies. There will be 2 novel biological products (DNA plasmid containing HA and M2e, and glycolipid of choice). We have already contacted the Center for Drug Evaluation to set up a window of communication to discuss issues relevant to future filing of IND.

Reviewer 2:

1. 第一年的目標（1）生產 H5 DNA 疫苗，進行小鼠免疫原性試驗，並生產 α-Gal-Cer 以測試 α-Gal-Cer 輔助 H5 疫苗的佐劑效能（2）.生物技術開發中心量產 GMP 規格 20 萬劑量的 H5 DNA 疫苗與 α-Gal-Cer 佐劑似乎都沒達到。

在計畫書中原訂「生物技術開發中心量產 GMP 規格 20 萬劑量的 H5 DNA 疫苗與 α-Gal-Cer 佐劑」，於計畫申請審查時已考量下列因素被刪除：

(1) 生技中心主要的工作在於實行建立製程技術，以利 DNA 疫苗之開發，並作為未來 GMP 藥廠量產之準備，因此審查委員建議有關生技中心後續產製 DNA 疫苗部分，只進行至技術平台之建立。

(2) 所研發之 DNA 疫苗如尚未經過藥理、毒理等測試，未得衛生署藥政處核准，依現行法規不得逕行 GMP 規模之疫苗生產。

(3) 生物技術開發中心目前尚無 GMP 量產規格之設備，無法進行 20 萬劑量 DNA 疫苗的量產，仍需仰賴符合 GMP 且具相當規模之藥廠來進行。

後因作業疏失，計畫書並未完全修正，在「實施方法及進行步驟」仍保留此文字，而在年度研究成果報告中亦誤植，請審查委員見諒。

2. 圖二的（a）和（b）是一樣的沒有重量 loss 的比較圖:

We apologize for the inadvertent clerical error. The correct figure 2b is now shown in the revised report.

3. 測試 glycolipid 的佐劑效果如果用 HA 和 Me2 當抗原測試，且只要抽兔子的血測試 HI 或中和效應，就可初步了解其效果，無需先進行病毒攻擊試驗，如此一來才能加快計畫執行的速度

We agree entirely with the suggested testing of HI and neutralizing activities of the antisera induced by HA and M2e DNA vaccine. However, with the reassorted NIBRG14 and the avian H5N1 strain from Damsui being the only available H5N1 virus strains in Taiwan, it is not feasible to test the scope of cross reactivities of antibodies generated by our vaccine.

Recently, we have circumvented this problem by constructing pseudotyped virus-like particles (VLP) with H5 derived from different strains of H5N1. We found that the antisera generated in mice after immunization with H5 DNA vaccine were able to neutralize several different VLP including Vietnam 1203, Vietnam1194, Qing Hai, Hong Kong2003 Indonesia, Tamsui (Kingmen) strain. This new finding is now included in the 2006 Annual Progress Report. However, to determine whether our H5 vaccine can indeed protect mice from a broad spectrum of H5N1 challenge, it is imperative that H5N1 viral strains representative of different clades are available in Taiwan.

4. 本計劃為新疫苗開發,可分為 a.雙效疫苗;b. 醣脂佐劑兩部分.成為上市新藥的挑戰非常高.建議建立工作項目：甲、所需測試具代表性的流感病毒株清單及如何處理/安置這些病毒；乙、雙效疫苗與醣脂佐劑應先分開探討其作用,先建立基本數據再看兩者的加乘作用；丙、如醣脂佐劑本身有作用,則探討其廣泛的應用性；丁、現階段應開始計畫未來人體臨床試驗的設計。

甲、 所需測試具代表性的流感病毒株清單及如何處理/安置這些病毒

(1) Indo/05/2005(H5N1)/PR8-IBCDC-RG2: available from CDC in USA and Taiwan. MTA is signed. This reassorted virus will be used in the P2-plus facility at the Genomics Research Center.
(2) NIBRG-23, a reassorted H5N1 from A/turkey/Turkey/1/2005: available from National Institute for BiologicalStandards and Control, England. MTA is being signed. This reassorted virus will be used in the P2-plus facility at the Genomics Research Center.
(3) A/HK 1997 H5N1,A/HK 2003 H5N1 and A/VN 2004 H5N1: These are 3 pathogenic H5N1 strains, to be provided thru courtesy of Dr. David Ho, pending approval procedures in Taiwan. These viruses will be stored and tested in the certified P3 facility at 農委會家畜衛生試驗所, in collaboration with us.

乙、雙效疫苗與醣脂佐劑應先分開探討其作用,先建立基本數據再看兩者的加乘作用:

We agree with the suggestion and this is exactly what we had proposed in our original research plan.

丙、如醣脂佐劑本身有作用,則探討其廣泛的應用性.

We have indeed provided data in our annual report showing the potent immune-modulating effects of the newly synthesized glycolipids, in vitro and in vivo, for human and mice, respectively. We also provided evidence demonstrating the adjuvant activities of glycolipids for protein and peptide antigens. In addition, we have evidence that these glycolipids have strong anti-cancer effects in animal studies, which is beyond the scope of this study, and therefore not included in the report.

丁、現階段應開始計畫未來人體臨床試驗的設計.

The overall goal of the proposed study is to generate all the necessary data for IND filing at the end of 3rd year. Thus, a phase I protocol will be developed during year 2009 when we generate the necessary information regarding the type of H5/ M2 DNA vaccines, optimal dose schedule, selection of best glycolipid. We will develop the clinical protocol sooner if our progress exceeds the expected time line.

Reviewers' Suggestions after oral presentation on December 6, 2006:

1. *Th2-biased glycolipids may have better adjuvant activity than Th1 biased compounds for DNA vaccine, leading to better anti-viral efficacy of the vaccine.*

   Although humoral immunity is crucial for the defense against influenza infections, cellular immunity may also be important. We thus plan to evaluate the adjuvant activities of both Th1 and Th2 biased glycolipids for H5 DNA vaccine.

2. *Consider the use of CpG as a positive control for evaluating adjuvant activity of glycolipids:*

   We had planned to use alum as a positive control for adjuvant activity. As suggested by the reviewer, we may also consider the use of CpG oligodeoxynucleotides as a positive control. However, a potential drawback is that CpG motifs may trigger deleterious autoimmune reactions under certain circumstances.