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以輪狀病毒非表面蛋白質做為標的之疫苗研究

Study of rotavirus non-surface proteins as the target for vaccine development

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一、中文摘要

輪狀病毒是引起小兒嚴重脫水性腸胃炎的一個最重要的致病原，因為此感染症明顯的多且無法因改善衛生而加以預防，疫苗的發展格外的迫切需要。一種四合一的輪狀病毒疫苗曾於 1998 年在美國上市並開始施打，但不幸因與腸套疊之發生相關，而於次年宣佈撤回。由表面蛋白質之基因變異性或抗原性之差異，輪狀病毒可分為至少 19 種 P 基因型或 14 種 G 血清型。VP6 蛋白質是病毒中層蛋白質殼的主要成份，依其抗原性大多數的人類輪狀病毒可分為兩種亞群。NSP4 是一種穿膜的非結構性蛋白質與病毒的組裝有關，並被認為是一種腸內毒素。分泌型的 IgA 抗體與對抗黏膜致病原的保護力有關。曾有小鼠的研究證實不具中和能力的 VP6 IgA 單株抗體可以預防初次感染，並可減輕小鼠的輪狀病毒感染。此可能與 VP6 IgA 抗體在分泌過程中穿過上皮細胞時可抑制病毒的生長有關。因 VP6 及 NSP4 在輪狀病毒的結構或功能上極重要，又比輪狀病毒的表面蛋白質保守性高，故本計劃以腺病毒載體分別表現此二蛋白質，輔以細胞激素或免疫佐劑，期以發展出可引發黏膜免疫力且具較廣效型的疫苗。本計畫之主要目標即是要研發出帶有 VP6 或 NSP4 基因之腺病毒載體疫苗，可以對抗不同血清型別之輪狀病毒感染，並可引發黏膜免疫力。

本計畫已分別將 VP6 蛋白質及 NSP4 蛋白質之基因擇殖入大腸桿菌表現載體。全長之 NSP4 基因，無法表現出蛋白質，去除 N 端部分之 $NSP4_{86\text{-}175}$，可以順利表現出蛋白質。全長之 VP6 基因，無法表現出蛋白質，去除 N 端部分之 $VP6_{197\text{-}397}$，是否可以順利表現出蛋白質尚在測試中。此外，已完成構築四種重組腺病毒，分別含有 NSP4 full length，$NSP4_{86\text{-}175}$，VP6 full length，$VP6_{197\text{-}397}$ 等基因片段，並已完成溶斑純化之工作，經螢光免疫試驗或酵素免疫反應確定均可表現出蛋白質。

關鍵詞：輪狀病毒，腺病毒載體， VP6 蛋白質，NSP4 蛋白質，黏膜免疫力，疫苗

Abstract

Rotavirus infections are the most important cause of severe, dehydrating gastroenteritis among children worldwide. Because of the significant disease burden and the impossibility to be prevented by improvements in hygiene and sanitation, vaccines against rotavirus are urgently needed. A tetravalent rhesus-based rotavirus vaccine was approved in the U. S.

in 1998 and withdrawn in the following year for its association with intussusception. Based on the genetic variation or the antigenicity of the surface proteins, VP4 and VP7, rotaviruses can be classified into at least 19 P genotypes and 14 G serotypes. VP6 protein is the major component of the middle-layer capsid. Based on the antigenicity of VP6, most of human rotaviruses can be grouped into two subgroups. NSP4 protein is a nonstructural transmembrane protein important for viral assembly and is suggested as an enterotoxin. Secretary immunoglobulin A (IgA) antibodies are associated with protection against mucosal pathogens. It has been shown that non-neutralizing VP6-specific IgA monoclonal antibodies were capable of preventing primary infection and resolving murine rotavirus infection. It has been suggested that the VP6-IgA antibodies inhibited viral replication during the process of transcytosis through the epithelial cell. The VP6 and NSP4 proteins are important for viral structure or replication and are more conserved than the surface proteins. Adenovirus expressing VP6 or NSP4 protein formulated with cytokines or adjuvants is intended to develop more cross-protective vaccines capable of inducing mucosal immunity.

We have cloned $NSP4_{\text{full length}}$, $NSP4_{86\text{-}175}$, $VP6_{\text{full length}}$, and $VP6_{197\text{-}397}$ genes into DNA vectors for protein expression in E. coli system. Full length NSP4 and VP6 genes could not be expressed. However, NSP4 gene with deletion of the N-terminal region was successfully expressed. The recombinant adenoviruses containing one of the four genes were constructed and plaque-purified, and the expression of protein was confirmed by immunofluorescence assay and/or ELISA.

Keywords: Rotavirus, Adenovirus vector, VP6 protein, NSP4 protein, Mucosal immunity, Vaccine

二、緣由與目的

Rotavirus has a characteristic triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and an inner core layer (15). The genome consists of 11 double-stranded RNA segments and each segment encodes one or more polypeptides (15, 19). VP (virus protein) 1, 2 and 3 form the core of the virus. VP6 forms the intermediate layer. VP4 and VP7 form the outer capsid, and are responsible for virus attachment and entry into susceptible cells. Six non-structural proteins (NSP1-6) are found only in the infected cell. They are involved in viral replication (NSP1 to 3, 5, 6) and morphogenesis (NSP4) (154).

Rotaviruses can be subdivided into groups, subgroups, and types by its antigenicity (22). The group- and subgroup-specific antigens are present on VP6. There are seven groups (A-G). Group A rotaviruses cause most human infections, and these can be subgrouped into mainly I and II. Rotaviruses can be classified into P and G types on the basis of the two surface proteins, VP4 and VP7. Antibodies to these proteins are neutralizing both in vitro and in vivo (15). Serotypes G1-G4 are the predominant types associated with severe disease in human (37). The P genotypes primarily associated with human infections are P[4], P[6] and P[8] (17,22).

VP6 protein is the major structural protein of rotavirus and is encoded by gene

segment 6 in group A rotavirus. VP6 protein contains 397 amino acids and constitutes 51% of virion protein (15,22). It plays a key role in virion structure because of its interaction with both the outer capsid proteins and the core protein VP2. It is highly antigenic and immunogenic (14). It is generally accepted that antibodies to rotavirus group antigens are not neutralizing. However, IgA monoclonal antibodies directed against VP6 have been shown to protect mice from rotavirus infection and clear chronic infection in SCID mice (9,16). It has been suggested that the VP6-IgA antibodies inhibited viral replication during the process of IgA transcytosis through the epithelial cell (9).

Non-structural protein NSP4, encoded by gene 10 in group A rotaviruses, is a transmembrane protein of the ER (15). NSP4 has been studied extensively for its importance in virus morphogenesis and in virulence by functioning as an enterotoxin (4,41). The C-terminal cytoplasmic domain (amino acids 161 to 175) plays a role in viral assembly by acting as an intracellular receptor on the ER membrane (2,35,36). NSP4 has been implicated in rotavirus virulence (20). Free NSP4 and a 22-amino acid peptide (amino acids 114 to 135) were both capable of inducing a dose-related diarrhea when administered intraperitoneally or intraduodenally to 6- to 10-day-old mice (4). NSP4 gene is well conserved as compared to VP7 and VP4 genes. Sequence analysis of NSP4 genes has revealed that the majority of human rotaviruses can be classified into two genogroups, genogroup A mainly containing strains of G2 type, genogroup B containing strains of other G types (15,30).

Rotavirus infections are the most important cause of severe, dehydrating gastroenteritis among children worldwide (22). Because of the significant disease burden associated with rotavirus, and the observation that rotavirus is not likely to be prevented by improvements in hygiene and sanitation, efforts to develop vaccines against rotavirus have been under way since the early 1980s (8). The first rotavirus vaccine, a tetravalent rhesus-based rotavirus vaccine (RRV-TV), was approved by the Food and Drug Administration, the United States in 1998, and was included in the 1999 US schedule for routine childhood immunizations. Unfortunately, because of the association between RRV-TV and intussusception, this vaccine has subsequently withdrawn in the same year from the vaccination program (10,11). A variety of alternative approaches to new vaccines are being pursued, including a live human strain, Jennerian vaccines, new human-animal reassortants, parenterally administered antigens and DNA vaccines (26).

Mucosal immune responses are characterized by production of antibodies of the IgA isotype by plasma cells lying in submucosal tissues (28). The Th2-type cytokines, IL-5 and IL-6, significantly enhance IgA synthesis in murine Peyer's patch B-cell cultures (5,6,18,34). Recombinant vaccinia viruses that encode IL-5 were applied to mice intra-nasally and were shown to have boosted IgA responses to co-expressed HA antigen (32). A number of strategies are being employed to enhance mucosal immunity including: (1) mucosal adjuvants such as choloera toxin (21); (2) microparticles especially for oral delivery. These microparticles are generally biodegradable and can even be used for

pulsatile release. Both polylactide-glycolide, and alginate particles have been shown effective, especially for oral delivery (72,23,29); (3) DNA vaccines (13,24); (4) transgenic plants (1, 40); and (5) live vectors (31,39). One of the first licensed recombinant vaccines employed in vaccinology included vaccinia virus carrying the gene encoding rabies virus glycoproteins. This vaccine is successfully used in wildlife against rabies (27).

Adenoviruses that replicate in the gut have great potential in eliciting mucosal immune responses and were proven very effective and safe as vectors for viral vaccines. Most of the adenoviruses enter the host via mucosal surfaces and, therefore, replicate, at lease initially, in mucosal sites of the respiratory or gastrointestinal tracts (3). Therfore adenoviruses are attractive as vectors for delivering vaccines to mucosal surfaces. Recombinants using adenovirus as vector have been described and used for a variety of animal viruses (12,25,33). A study using replication-defective adenovirus recombinant to induce immune responses against rabies has shown that it is possible to induce good mucosal immune responses using non-replicating adenoviruses by immunizing intranasally (38).

In this research we will develop rotavirus vaccine using adenovirus as the vector to express rotavirus VP6 and/or NSP4 protein. The constructs for expressing cytokine fusion proteins (IL-5 and IL-6) will also be generated to enhance the immune responses. We anticipate that the inherent cytokine activity of the expressed fusion protein will help inducing immune responses, and hopefully, adenovirus vector vaccine will thus be developed, that will be able to induce high level of mucosal IgA antibody to rotavirus proteins that will be cross-protective to different types of rotavirus infection. VP6 and NSP4 proteins will also be expressed in E. coli expressing system and used for immunization. The adjuvants capable of inducing mucosal immunity will be co-administered with the protein antigens.

三、結果與討論

The NSP4 and VP6 genes were amplified from RNA extract of rotavirus samples by RT-PCR. The PCR products were cloned into T vector using a TA cloning kit. The two genes were further modified to delete the N-terminal regions ($NSP4_{86\text{-}175}$ and $VP6_{197\text{-}397}$). These genes were transferred to protein expression plasmid vector pRSET. The full-length NSP4 and VP6 genes could not be expressed. This was probably related with the hydrophobic domains. However, the truncated NSP4 protein was successfully expressed in E. coil. The expression amount was also satisfactory in this system. The protein expressed was confirmed by NSP4 antiserum. All the constructs, including $NSP4_{full\text{-}length}$, $NSP4_{86\text{-}175}$,$VP6_{full\text{-}length}$, and $VP6_{197\text{-}397,}$ were further cloned into adenovirus vectors. The recombinant adenoviruses were successfully produced and plaque-purified in 293A cells. Using PCR showed that the VP6 or NSP4 gene was indeed present in the adenoviruses. The protein expression was confirmed by immunofluorescence assay and/or ELISA. The expression of VP6 by inoculation of $rADV/VP6_{full\text{-}length}$ in 293A cell, could be observed starting from 24 h, and reaching the maximum at 72 h, evenly distributed in the cytoplasm. The expression of NSP4 by $rADV/NSP4_{full\text{-}length}$ could be observed after 48 h with small particles probably associated with ER membrane. The expression of truncated NSP4 by $rADV/NSP4_{86\text{-}175}$ could be observed at 24 h after infection along the cytoplasm membrane. The amount of immunofluorescence became inevident after 48 h post-infection. This was probably due

to the secretion of protein from the cells. Large amount of recombinant adenoviruses will be produced for immunization.

四、計畫成果自評；

本計畫已完成 NSP4 及 VP6 基因的擇殖、修飾，並已成功的表現 NSP4 蛋白質，提供後續實驗抗體偵測之用。亦構築完成含有 $NSP4_{full\text{-}length}$、$NSP4_{86\text{-}175}$、$VP6_{full\text{-}length}$、$VP6_{197\text{-}397}$ 等之重組腺病毒，分別可表現蛋白質，但表現之量仍嫌較低，如何增進其表現之量，是有待努力的方向。

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