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総括研究報告書

食べる腸管感染ウイルスワクチンの開発

主任研究者　武田直和

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研究要旨　食べるウイルスワクチンの開発を目的として、E 型肝炎ウイルス構造蛋白を産生するトランスジェニックトマトを作製した。組換えバキュロウイルスで発現した E 型肝炎中空粒子をカクニイザルに経口投与したところ、マウスと同様の抗体産生が誘導され、チャレンジに対し感染防御と発症阻止が観察された。

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A.　研究目的

ワクチンによる予防が可能な感染症の中で、子供のウイルス性感染症、特に腸管感染ウイルスによる下痢症と肝炎は、開発途上国において常に年間死亡率の上位を占めている疾患である。本研究では腸管感染ウイルスのうち、わが国では生ガキによる集団食中毒で問題になる SRSV、開発途上国の経口伝播型急性肝炎の主要病原体であり、近年わが国へも輸入感染症として持ち込まれるケースが多い E 型肝炎ウイルスについてその構造蛋白を発現するトランスジェニック植物を作製し、食用ワクチンとしての有効性を評価することを目的とする。

SRSV には多数の血清型があるため 10 種以上の抗原性を持つ多価ワクチンを開発する必要があるが、植物は組み込める遺伝子数に事実上制限がないのでこの目的には最適である。E 型肝炎は診断法の確立と共に早急に予防対策を講ずる必要がある。トランスジェニック植物の開発は、国際社会、特に日本を含む先進国に対して提言された CVI （Children ɪ s Vaccine Initiative）に対する最も優れた解答の一つである。また現在 WHO が進めている子供ワクチン計画のポリオ、マシン撲滅に続く疾患対策にも、これを推進する上で極めて有効な手段を提供することができる。

本研究が対象としているウイルスでは、いずれもトランスジェニック植物体内でウイルス遺伝子を有しないウイルス様中空粒子として産生されるため、通常の経口ワクチンと異なり投与されたウイルスが増殖することは全くない。したがって AIDS 患者のように免疫不全であったり、免疫欠損の個体にも投与することができる。本研究の最終目的であるトランスジェニックバナナは、上記に示したワクチンの条件をすべて満足する理想的な食用ワクチンと考えられる。

B.　研究方法

1) E 型肝炎ウイルス（HEV）構造蛋白を産生するトランスジェニックトマトの作製

HEV はエンベロープを持たない直径約 27nm の小型の球形ウイルスである。ゲノムは約 7.2kb のプラス一本鎖 RNA で、3’末端にポリアデニル酸をもつ。3’末端に位置する約 2.0kb の ORF2 は約 660 アミノ酸、分子量 72kDa の構造蛋白をコードする蛋白である。1986 年ミャンマーで分離された HEV を用い、アミノ末端 111 アミノ酸を欠失させた ORF2（△N111）、およびアミノ末端のほかにカルボキシ末端 52 アミノ酸を欠失させた ORF2（△N111△C52）をバイナリーベクタ

ーにクローン化し、アグロバクテリアを形質転換した。NV 同様トマトの葉に感染後、形質転換体を抗生物質で選択し、幼植物体を得た。

2) 発現蛋白の解析

幼植物体の葉を採取し、プロテアーゼ阻害剤を含む PBS(-)を加えてダウンスホモジナイザーで破砕して 10%ホモジネートを調製した。蛋白を SDS-PAGE で分離後、ニトロセルロース膜に転写した。常法に従いてウエスタンブロット法で発現蛋白を検出した。一次抗体には HEV VLP をウサギに免疫して作製した高力価血清を用いた。また抗体 ELISA 法でホモジネート中の発現蛋白を検出した。

3) 組換え E 型肝炎ウイルス中空粒子を経口投与したカククイザルの免疫応答

HEV の ORF2 の N 末端から 111 アミノ酸を欠失させたフラグメントを pVL1393 にクローニングし、組換えバキュロウイルスを作出し、昆虫細胞 Tn5 細胞に感染させた。培養上清から、浮上密度 1.285g/cm3、直径約 23-24nm のウイルス様中空粒子が大量に得られた。7 日目の培養上清を 1000x g で遠心して、組換えバキュロウイルスを除き、塩化セシウム平衡密度勾配遠心で純度の高い粒子を得た。カニクイザルに精製した HEV VLP を経口投与し、マウス同様血中 IgM、IgG および腸管 IgA の産生を ELISA 法で測定した。

C.　研究結果

1) E 型肝炎ウイルス構造蛋白を産生するトランスジェニックトマトの作製

アミノ末端 111 アミノ酸を欠失させた△N111、およびアミノ末端のほかにカルボキシ末端 52 アミノ酸を欠失させた△N111△C52 をバイナリーベクターにクローン化し、アグロバクテリアを形質転換した。トマトの葉に感染後、形質転換体を抗生物質で選択し、△N111 で 15、△N111△C52 で 22 の幼植物体を得た。葉をホモジネートし ELISA で発現蛋白量を測定したところ 0.5-3.0ng/ug の発現量であった。果実に 0.7ng/ug の発現量を持つ個体を得た。

2) 組換え E 型肝炎ウイルス中空粒子を経口投与したカククイザルの免疫応答

マウスにおける至適量を基礎にマウスとカニクイザルの体重比から一頭当たり 10mg の VLPs をミカン果実（2-3 房）に注入し、予め絶食しておいたサル 2 頭に与えた。この方法でサルは確実に摂食することは確認済みである。初回免疫日を 0 日とし、これを確実に行なうために 1 日めに同量の VLPs を再度経口投与した。その後 14、28、42 日に追加免疫を行なった。毎週採血と採便を行い、血中 IgM、IgG および便中の IgA 抗体を VLPs を抗原に用いた ELISA で検出した。血中 IgM 抗体はマウスでみられたような顕著な上昇は観察されなかった。IgG 抗体は 2 頭とも 3 週目には上昇し始めたが、抗体価はマウスに比べ低かった。80 日を経過した時点で、腸管 IgA 抗体の検出はできなかったので、84 日に追加免疫をおこなった。その結果、血中 IgG 抗体の急激な上昇がみられた。感染カニクイザルの糞便から調製し、感染性 HEV を確認してある乳剤でチャレンジを行ったところ、明らかに感染防御と発症阻止が観察された。

D.　考察

本年度は HEV 構造蛋白を産生するトランスジェニックトマトが得られた。先に米国でおこなわれた小型球形ウイルスの構造蛋白をタバコで発現する実験では、組換えバキュロウイルスで産生される VLP と形態学的にも免疫学的にも差異のない粒子が植物体内で発現されている。したがって、今回用いたトマトでも粒子の産生が十分期待できる。トマトは生で食べることができるので食べるワクチンとしては有望である。

ウイルス様中空粒子の腸管免疫誘導能を HEV に感受性を示すカニクイザルで試験した結果、血中に発症阻止と感染防御能を有する抗体の産生が確認された。組換えバキュロウイルス発現系は優れた系ではあるが、ヒトへの応用にはより大量の抗原が必要であり実用的ではない。トランスジェニック植物が求められる所以であるが、さらなる発現効率の改良が必須である。

E.　結論

組換えバキュロウイルスで発現した HEV VLP

を経口投与することによって、発症阻止能をカニクイザルで誘導できた。HEV 構造蛋白を産生するトランスジェニックトマトの結果が待たれる。

F. 健康危険情報

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H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

Plant-based Oral Vaccine for Hepatitis E Virus
Report for Society of Japanese Pharmacopoeia, 4/17/01

Introduction

We have used plants for expression and delivery of recombinant subunit vaccine antigens, including Norwalk virus capsid protein (NVCP; Mason et al., 1996). With Carol Tacket at Center for Vaccine Development (Baltimore, MD) we performed clinical trials using potatoes expressing *E. coli* LT-B or NVCP(Tacket et al., 1998; Tacket et al., 2000). Both trials showed very promising results, with 19 of 20 volunteers showing immune responses against NVCP delivered by ingestion of raw transgenic potatoes containing approximately 500 μg antigen per dose. Our studies with NVCP expression in tobacco and potato cells demonstrated that subunits assembled to form virus-like particles (VLP) very similar to those obtained with baculovirus-infected insect cell expression, although up to 75% of the antigen was present as monomers or partially assembled aggregates (Mason et al., 1996). VLP assembly may be important for obtaining stability against acid and protease-mediated degradation in the stomach, as well as for presentation of conformation-dependent epitopes that may be needed for effective virus neutralization.

Hepatitis E virus capsid protein (HEVCP) has been expressed in baculovirus-infected insect cell system and shown to assemble virus-like particles (VLP; Li et al., 1997, Xing et al., 1999). These VLPs were highly immunogenic and antigenically similar to authentic HEV virions, and thus represent a good candidate for a subunit vaccine. Our goal is to create transgenic plants that express HEVCP in edible tissues, in order to obtain an economical oral vaccine. We described in the 2000 report the construction of plant expression cassettes containing the HRV ORF2, which encodes the capsid protein, and use of these vectors to create transgenic potato and tomato plants. We showed expression in potato leaf up to 0.3% of total soluble protein, which is similar to NVCP expression (Mason et al., 1996). In this report we describe expression of HEVCP in potato tubers, and processing of the transgenic tubers by extraction and freeze-drying to obtain dry powder to be used by Dr. Takeda in animal feeding experiments.

Methods, Results, and Discussion

<u>HEVCP expression in potato tubers</u>. Ten independent transgenic lines of HEV101 and 6 lines of HEV110 (refer to constructs in 2000 report) were grown to maturity in the greenhouse and tubers were harvested and washed. ELISA for HEVCP was performed on tuber extracts as described for leaf extracts (2000 report). Accumulation of HEVCP in tubers varied from about 5 to 30 μg per gram fresh tuber, depending on the transgenic plant line (**Figure 1**). This compares well with the expression of NVCP in potato tubers (Mason et al., 1996) and is better than expression of *E. coli* LT-B protein in potato (Mason et al. 1998). We further compared the expression levels with the yield of tubers for each line and plotted the results in **Figure 2**. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 grams per pot. We used the data of Figure 2 to select lines HEV101-16, HEV101-23, HEV110-27, and HEV110-28 for highest yields of recombinant protein.

Figure 1. Expression of HEVCP in tubers of transgenic potato lines. HEV101 (10 lines) or HEV110 (6 lines) tubers were extracted and assayed by ELISA for HEVCP. Error bars indicate standard error for 3 different tubers from the same line.

Figure 2. HEVCP expression vs. tuber yield for different transgenic potato lines.

Studies on processing transgenic potatoes. We extracted and freeze-dried potatoes expressing HEVCP in order to evaluate effects of different conditions on recombinant protein as measured by ELISA. Figure 3 shows that HEVCP survives well when extracts are incubated for 30 minutes at up to 37 C, but is greatly reduced if the extracts are incubated at 60 C.

Figure 3. Effect of temperature on HEVCP in tuber extracts. Transgenic tubers of 2 different lines were homogenized with water or buffer and incubated at various temperatures for 30 minutes before clearing by centrifugation and assay by ELISA for HEVCP.

Figure 4. Effects of sodium ascorbate concentration on HEVCP in tubers extracted in water (left) or PBS buffer (right).

Since oxidation of tubers can cause browning and possible deleterious effects on proteins, we evaluated different concentrations of the antioxidant sodium ascorbate on transgenic potato tuber extracts. We found that HEVCP levels were indeed lower when the tubers were extracted with water alone, but that increasing levels of sodium ascorbate preserved the antigen in the tuber extract (**Figure 4**). Interestingly, the effect of ascorbate was reduced if the tubers were extracted in PBS buffer, but we still observed some enhancement of antigen level with increasing ascorbate up to 160 mM. We then tested the effects of freeze-drying after extraction in different buffers on HEVCP content. **Figure 5** shows the results of this study. We found the best preservation of antigen using PMSF (protease inhibitor) with ascorbate, but the differences were minor.

Figure 5. Effects of freeze-drying on HEVCP content of potato tubers. Transgenic potato tubers were homogenized in different buffers and freeze dried. The primary extract (control) was assayed by ELISA and the freeze-dried powder was extracted and assayed. "Processed" material was completely dried during the process, while "Incomplete" material did not dry completely.

We selected tubers of lines HEV101 and HEV110 from the tubers harvested and stored at 4 C for freeze-drying and sending to Dr. Takeda for animal feeding studies. In order to avoid the use of PMSF (proteinase inhibitor) and PBS buffer, we extracted the tubers in 100 mM sodium ascorbate and freeze dried them. The powder was sent to Dr. Takeda for evaluation in animals.

We planted tubers of selected potato lines (HEV101-16 and –23), grew 10 plants of each line, and recently harvested the tubers. They will be assayed for HEVCP and approximately 12 kg will be freeze dried for shipping to Dr. Takeda by May 1, 2001. We are now planting tubers of lines HEV110-27 and –28, and anticipate harvest, freeze-drying, and delivery to Dr. Takeda by about September 1, 2001. These potato powders will be used for immunogenicity studies in primates.

<u>HEVCP expression in tomato</u>. Studies with tomato have been much less successful than those with potato. We have not yet been able to regenerate tomato lines expressing more than about 0.07% of total soluble protein in leaf tissue, and the fruit expression in these few lines has been at most 0.25 μg per gram fruit. It is not clear why tomato shows low expression, but the poor efficiency of transformation suggests that the HEVCP may somehow hinder growth of tomato plants. Thus we will construct a new expression vector using a fruit-specific promoter, which may allow greater transformation efficiency by avoiding constitutive expression.

Future plans

We will soon assay expression of HEVCP in potato tubers from the larger scale planting of HEV101 and HEV110 lines. The harvested tubers will be processed by freeze-drying and the powder shipped to Dr. Takeda for immunogenicity analysis by feeding to animals. We will continue studies on VLP assembly in plant tissues in order to determine why VLPs appear to be unstable in plant extracts, even thought the capsid protein is quite stable. If the monkey feeding trials show promising results, we will produce more potato material for anticipated human trials. We will construct a fruit-specific expression vector for HEVCP using the tomato E8 promoter, generate transgenic tomato plants, and evaluate antigen expression in ripening fruit. We have used this promoter for expression of *E. coli* LT-B protein in tomato at levels of up to 15 μg per gram fruit.

Timeline:

5/01-6/01 Process HEV101-16 and -23 potato tubers and deliver potato powders to Dr. Takeda for monkey feeding trials.

6/01-7/01 Construct E8 promoter-driven fruit-specific expression vector for tomato; begin tomato transformation.

6/01-9/01 Evaluate VLP assembly/stability in fresh and freeze-dried potato tubers.

8/01 –9/01 Process HEV110-27 and -28 potato tubers and deliver potato powders to Dr. Takeda for monkey feeding trials.

9/01-10/01 Screen E8-HEV tomato transformants by ELISA for leaf expression of HEVCP; select lines for greenhouse production.

10/01-1/02 Grow E8-HEV tomato plants, assay fruit expression collect seeds.

1/02-2/02 Experiment with freeze-dry processing of transgenic tomatoes to evaluate antigen preservation.

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Augmentation of Immune Responses to Hepatitis E Virus ORF2 DNA Vaccination by Codelivery of Cytokine Genes

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ABSTRACT

DNA vaccines encoding a viral structural protein have been shown to induce antiviral immune responses and provide protection against subsequent viral challenge. In the present study we show that DNA immunization with a plasmid expressing the hepatitis E virus ORF2 structural protein (pcDNA-ORF2) induced low levels of long-lasting antibody responses in the murine model. The use of plasmids expressing interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating-factor (GM-CSF) in conjunction with pcDNA-ORF2 enhanced the antibody responses generated by pORF-2. We further show that the immune responses generated by plasmid pcDNA-ORF2 can be boosted with virus-like particles composed of the ORF2 protein expressed through a baculovirus expression system.

INTRODUCTION

Hepatitis E virus (HEV) is the major etiologic agent of enterically transmitted non-A, and non-B hepatitis, now called hepatitis E (19). It is transmitted primarily by the fecal-oral route, with fecally contaminated drinking water being the most commonly documented vehicle of transmission. Although hepatitis E occurs in large outbreaks, HEV infection also accounts for about 30% of all acute sporadic hepatitis in children and adults in India and other endemic area (26). Virtually all cases of acute hepatitis E in nonendemic areas have been reported among travelers returning from high-HEV–endemic areas. Outbreaks of hepatitis E have occured over a wide geographic area, primarily in developing countries with inadequate environmental sanitation (26). In most hepatitis E outbreaks, the highest rates of clinically evident disease have been in young to middle age adults. Although protracted viremia up to 3 months has been observed, no evidence of chronic infection has been detected in long-term follow-up of patients with hepatitis E (26). The mortality rates are about 1%, except in pregnant women (18) and in the case of coinfection with other hepatitis viruses (1) where fulminant liver disease leading to high rates of mortality have been reported.

HEV is a spherical, nonenveloped virus with a positive-stranded RNA genome that has been cloned and sequenced from a number of geographically distinct isolates (2,4,14,28,29). The genome shows a high de-

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gree of nucleotide and amino acid sequence conservation and includes three open reading frames (ORFs) (28). The N-terminal ORF1 of approximately 5 kb is predicted to code for the putative nonstructural proteins, and the C-terminal region of approximately 2.4 kb carries ORF2 and ORF3. Of these ORF2 encodes the major capsid protein of HEV and ORF3 encodes a small protein of undefined function.

A vaccine against HEV, in addition to preventing sporadic hepatitis and seasonal epidemics in endemic areas, will be of much utility to pregnant women and travelers. Because of its poor growth characteristics in cell culture systems, traditional approaches such as a killed or live attenuated HEV vaccine are not feasible. Human B-cell responses to HEV in acutely infected humans are directed to the nonstructural as well as the structural proteins (16) and immunodominant epitopes have been mapped to the ORF2 and ORF3 protein (17). It has been shown that vaccination of monkeys with recombinant proteins that include either the full-length ORF2 protein (27) or only its C-terminal half (30) is capable of generating high-titer antibodies. Subsequent challenge of those immunized animals with HEV appeared to protect them from disease but not infection. Despite some degree of sequence heterogeneity and at least four genotypes of HEV present around the world (33), there appears to be a single serotype as evidenced from cross-neutralization assay (22). These studies suggest that a subunit vaccine based on the ORF2 protein could potentially provide protection against hepatitis E in humans.

Naked DNA or genetic immunization is a new technique in which plasmid DNA encoding either individual or a collection of antigens is directly administered to the host. Such immunization leads to expression of the delivered gene in host cells and its presentation to the immune system. This results in the induction of humoral as well as cytotoxic immune responses (11,21,31). DNA immunization has been shown to generate an immune response against several proteins derived from viruses, parasites, and bacteria (11) as well as induce protection against several infectious disease and cancer experimental model systems (31). It has also been reported that use of immunomodulatory molecules, such as cytokines, is effective in enhancing immune responses against proteins expressed *in situ* by DNA vaccination (32).

A DNA-based immunization strategy has previously been reported for HEV (13) in which inoculation of mice with a plasmid expressing ORF2 from the Burmese strain of HEV resulted in the generation of anti-ORF2 antibodies. In this study we report on the DNA immunization of mice with ORF2 from an Indian HEV isolate and modulation of the immune response by co-injection with expression vectors for two immunodulatory cytokines, interleukin-2 (IL-2), and granulocyte-macrophage colony stimulating factor (GM-CSF). We also report results from a DNA prime-protein boost strategy for HEV ORF2 immunization.

MATERIALS AND METHODS

Construction of expression vectors. The ORF2 of HEV was cloned in the *Eco*RV site of the polylinker region of plasmid pcDNA1 neo (Invitrogen, San Diego, California). This eukaryotic expression vector contains the cytomegalovirus early promoter/enhancer sequence and the polyadenylation and 3′-splicing signals from bovine growth hormone. Expression of the full-length ORF2 protein from this vector was established (S. Jameel, unpublished data) in transfected COS-1 cells followed by metabolic labeling and immunoprecipitation as described elsewhere (15). The pS2-S-IL2 clone, expressing mouse interleukin 2 (provided by M-H Tao, Taiwan) was digested with *Eco*R1 (to delete the pS2-S sequence) and religated to produce the pIL-2 expression vector. The GM-CSF clone expressing mouse GM-CSF (in plasmid pcDNA3) was kindly provided by Dr. O. Burrone (ICGEB, Trieste, Italy). Plasmid DNA was purified from transformed *Escherichia coli* DH5α cells by anion-exchange chromatography (Qiagen, Hilden, Germany). For experimental use, the DNA was reconstituted in sterile saline at a concentration of 1 μg/μL.

DNA-mediated immunization of mice. BALB/c female mice aged 6–8 weeks were obtained from National Institute of Nutrition (Hyderabad, India). The number of mice used per group ranged from 4 to 5. All mice were bled before injection for collection of preimmune sera. They were injected with 100 μg (or indicated amounts) of plasmid DNA in sterile saline into the left or right quadriceps. The DNA was injected using a 27-gauge needle fitted with a collar of polyethylene tubing that limited penetration to 2 mm. In some cases the animals were boosted with an additional dose of DNA or given an injection of virus-like

particles (VLPs) in saline. These VLPs were composed of the HEV ORF2 protein expressed in Tn5 insect cells using the baculovirus expression system (20).

Measurement of *in vivo* antibody production. Sera were collected by retro-orbital bleeding at various times following DNA injection and analyzed for the presence of anti-ORF2 antibodies. For anti-ORF2 detection, VLP-coated plates were used. Plates were coated overnight at 4°C with 100 μl of a 1 μg/mL solution of VLPs in carbonate buffer (pH 9.6). The plates were blocked with 200 μL of 5% powdered nonfat milk in phosphate-buffered saline (PBS) for 1 hour at 37°C. After washing, the primary antibodies (100 μL) were added at appropriate dilution (1 in 10 or 1 in 50) to the wells and incubation continued for 1 hour at 37°C. Same dilution of the preimmune sera served as the negative control. After washing with PBS containing 0.1% Tween 20, bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. Color development was done with *o*-phenylenediamine and the absorbance was measured at 492 nm. A serum sample was considered to be positive for anti-HEV when the optical density value was at least two times greater than that of preimmune sera with a cutoff value of 0.05.

For detecting isotype breakdown of the immunoglobulin G (IgG) antibody response, the reaction with test serum was followed by incubation at 37°C for 1 hour with goat antibodies to the appropriate murine isotypes. The dilution of serum was adjusted to obtain a comparable value for anti-ORF2 specific total IgG antibody. Color development and absorbance measurements were as described above. All the antibodies were obtained from Sigma Chemical Comapny (St. Louis, MO).

RESULTS

Effect of DNA dose on antibody responses. To determine the optimal amount of DNA necessary for vaccination, animals were given a single injection of 1, 10, 50, or 100 μg of pcDNA-ORF2 and the anti-ORF2 antibody levels were assayed at weekly intervals thereafter. The antibodies at week 3 postimmunization were of immunoglobulin M (IgM) isotype and at week 4 or thereafter were mainly of the IgG isotype. The antibody responses elicited by the plasmid DNA were dose-dependent, with higher doses of DNA producing an apparent increase in the anti-ORF2 antibody responses (Fig. 1A). Although lower doses of pcDNA-ORF2 (1 μg and 10 μg) produced some anti-ORF2 antibodies in the immunized animals, the absorbance values were very low (Fig. 1A). Therefore for futher studies, a 100 μg dose of pcDNA-ORF2 was used for immunization.

The longevity of the antibody response was determined after primary immunization with 100 μg of DNA followed by an equal booster dose after 4 weeks of initial injection. The mice were bled at regular intervals and the anti-ORF2 levels were determined. The antibody titers increased slightly after the boost and were maintained at peak levels for at least 24 weeks (Fig. 1B).

Effect of IL-2 coexpression on the anti-ORF2 response. An interleukin (IL)-2 expression plasmid was used to investigate the effects of this cytokine on the immune response elicited by ORF2 plasmid immunization. Three groups of mice (group A, B, and C) were inoculated with 100 μg of pcDNA-ORF2. Group B was coinjected with 100 μg of IL-2 plasmid at the same time as pcDNA-ORF2 while group C was injected with 100 μg of the IL-2 plasmid on day 4 after the injection of pcDNA-ORF2. Anti-ORF2 antibodies were detectable after 4 weeks of the primary injection in all three groups, at which time the mice were boosted with the respective plasmids, ie, group A received pcDNA-ORF2 and group B and C received same amounts of pcDNA-ORF2 and IL-2 expression plasmid (Fig. 2A). It was necessary to boost the animals with both the plasmids, ie, pcDNA-ORF2 and pIL2 in group B and C because boosting with pIL2 alone had no significant effect on the antibody titers. For 4 weeks after the first injection, the antibody levels in all three groups were similar. However, after 6 weeks of the first injection (ie, 2 weeks after the boost), antibody titers in the mice co-immunized with pIL-2 started increasing as compared to mice immunized with pcDNA-ORF2 alone. Sera samples from immunized mice were analyzed serially for up to 24 weeks. Antibody levels in group C immunized with pIL-2 at day 4 after pcDNA-ORF2 immunization peaked around 10 to 12 weeks while in group B which received pIL-2 along with pcDNA-ORF2 at day 0, the titers peaked around 16 weeks (Fig. 2A). It was interesting to note that the time of injection of the pIL-2 expression plas-

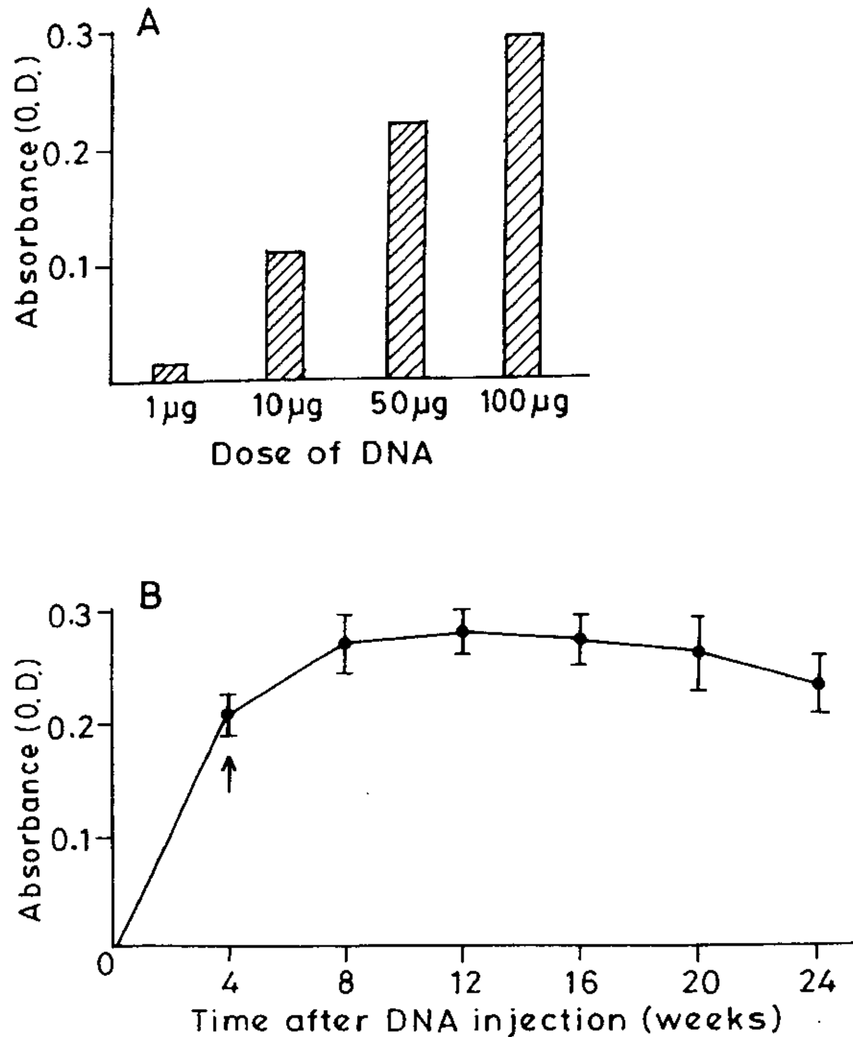

FIG. 1. **A:** Effect of DNA dosage on anti-ORF2 antibodies. Groups of five mice were given intramuscular injection of 1, 10, 50, or 100 μg of pcDNA-ORF2. Sera were collected and analyzed for the presence of anti-ORF2 antibodies by enzyme immunoassay (EIA). The figure shows the level of total immunoglobulin G (IgG) antibodies after 8 weeks of injection and is an average of duplicate absorbance (optical density, OD) values on pooled sera obtained after subtracting the values obtained with pre-immune sera (OD. of 0.05). **B:** Longevity of immune responses. Groups of five mice were injected with 100 μg of pcDNA-ORF2 and boosted 4 weeks later with same amount of DNA. Sera were collected at different time intervals and analyzed for the presence of anti-ORF2 antibodies with enzyme-linked immunosorbent assay (ELISA). The data are presented as mean $\pm$ SD for five animals per time point.

mid modulated the immune response. It was observed that augmentation of the response occurred when the IL-2 expression plasmid was administered 4 days after the ORF2 expression plasmid (Fig. 2A).

The anti-ORF2 IgG subclasses in the immune antiserum collected at 12 weeks postimmunization were determined for animals in group C. As shown in Figure 2B, the ratio of IgG_1 and IgG_{2a} antibodies was almost equal, suggesting that co-administration of an IL-2 expression plasmid enhanced both IgG_1 and IgG_{2a} antibodies (Fig. 2B). It was not possible to determine the IgG subclasses in the immune antiserum for animals injected with pcDNA-ORF2 alone (group A) because the titers were very low.

Effect of GM-CSF coexpression on the anti-ORF2 response. To study the effect of GM-CSF on antibody responses, 100 μg of the plasmid-encoding mouse GM-CSF was coinjected with pcDNA-ORF2. The

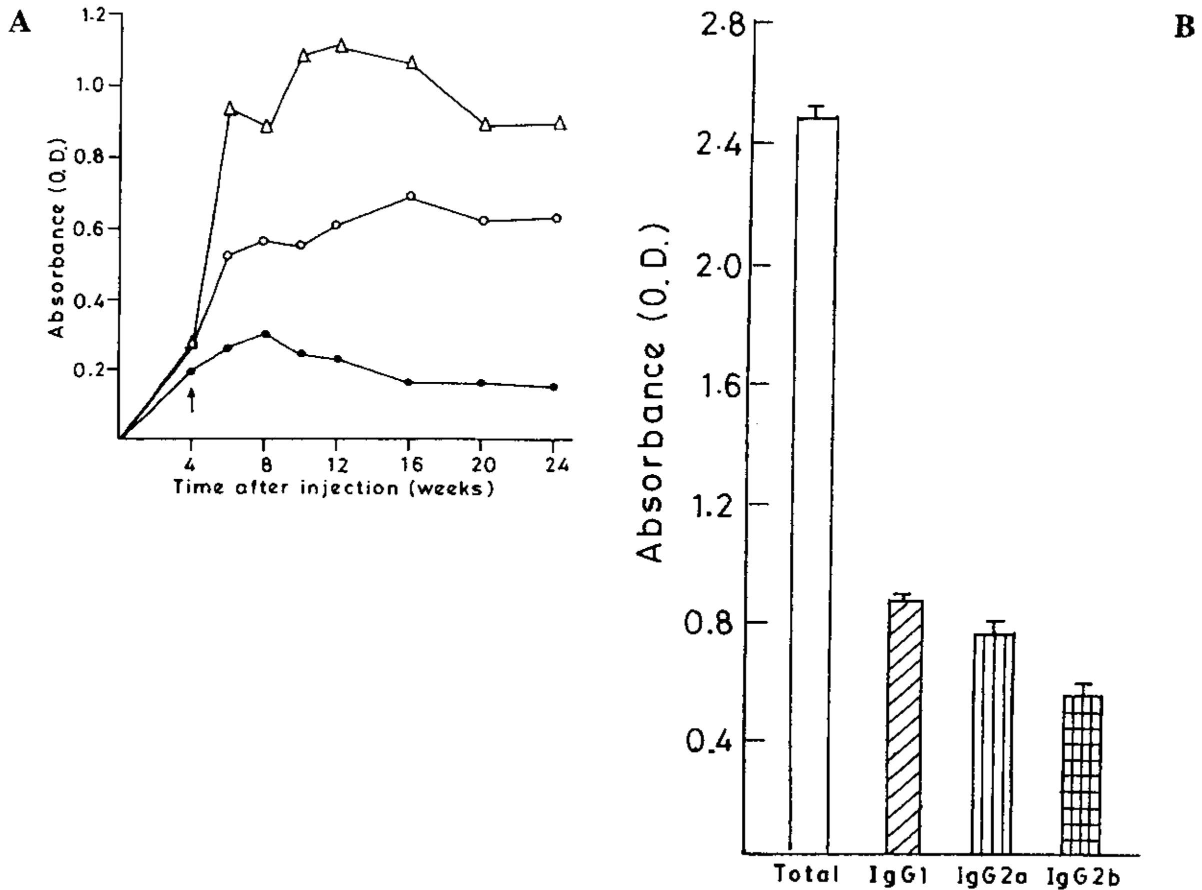

FIG. 2. Interleukin (IL)-2 expression plasmid coinjected at different times modulates the antibody responses. **A:** Groups of mice were injected with pcDNA-ORF2 (closed circles, group A), pcDNA-ORF2 and pIL-2 at day 0 (open circles, group B) and pcDNA-ORF2 and pIL-2 injected after 4 days of injection of pcDNA-ORF2 (triangles, group C). The arrow indicates the time of boost. The anti-ORF2 antibodies were measured in the pooled sera by enzyme immunoassay (EIA). Values shown are a mean of two separate experiments. **B:** Immunoglobulin-G (IgG) isotype profile in the sera of mice immunized with pcDNA-ORF2 and pIL-2 injected after 4 days of injection of pcDNA-ORF2 (group C of Figure 2). Anti-ORF2 specific total IgG, IgG_1, IgG_{2a}, and IgG_{2b} antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean ± SD for five animals.

control group received only plasmid pcDNA-ORF2. The sera were analyzed after 4 weeks of injection and antibodies to pORF2 were detectable in both groups. At this time, the two groups were boosted with the respective plasmids and the sera analyzed every 2 weeks thereafter for upto sixteen weeks (Fig. 3A). After 2 weeks of the booster, anti-ORF2 titers in animals receiving both pcDNA-ORF2 and pGM-CSF increased sharply and substantially as compared to the control group receiving only pcDNA-ORF2 (Fig. 3A). A boost with pGM-CSF alone had no effect on the antibody titers but when animals were boosted with both the plasmids, ie, pcDNA-ORF2 and pGM-CSF, the increase in antibody responses was observed. The effect of coinjection of GM-CSF, although very pronounced, was short lived, decreasing substantially 10–16 weeks postimmunization (Fig. 3A).

The anti-ORF2 IgG isotypes in the immune antiserum collected at 8 weeks postimmunization were measured in mice coinoculated with GM-CSF. As shown in Figure 3B, although the response was of a mixed type, there were more anti-ORF2 antibodies of the IgG_1 isotype compared to the IgG_{2a} isotype.

Boosting of humoral response. As shown earlier, boosting with DNA 4 weeks after the primary immunization resulted in a slight increase in antibody titers (Fig. 1B). It has been shown in other studies tha'

antibody responses elicited after DNA immunization can be augmented by administration of the antigenic protein (8). In order to study the effects of a protein boost on antibody responses, groups of mice were inoculated with pcDNA-ORF2 and given a booster of the same after 4 weeks of the first injection. Antibody titers were still low after the boost with DNA. These mice were then boosted, after 2 weeks of the DNA booster, with 10 μg of the ORF2 VLPs expressed using a baculovirus system (20). It was observed that the titers remained almost unchanged for 2 weeks after the boost with protein but after 4 weeks there was a sharp increase in these titers (Fig. 4A). The titers reached peak levels after 12 weeks of the initial inoculation followed by a sharp decline. The antibody levels were about half of the peak level at 16 weeks and decreased slowly after this time (Fig. 4A). In a control group of 10 animals, each immunized with only 10 μg of the ORF2 VLPs (in saline), the antibody response was weak and was observed for only 6 weeks. The optical density mean values after 4 weeks of immunization were 0.25 and the range of the value was 0.22 to 0.29. On the other hand, the antibody titers obtained after immunization of animals with 50 μg of VLPs in complete Freunds adjuvant were comparable to the titers obtained with DNA priming and protein boosting (data not shown).

The anti-ORF2 specific IgG subclasses in the immune antiserum collected at 12 weeks postimmunization were determined for animals primed with pcDNA-ORF2 and boosted with VLPs. As shown in Figure

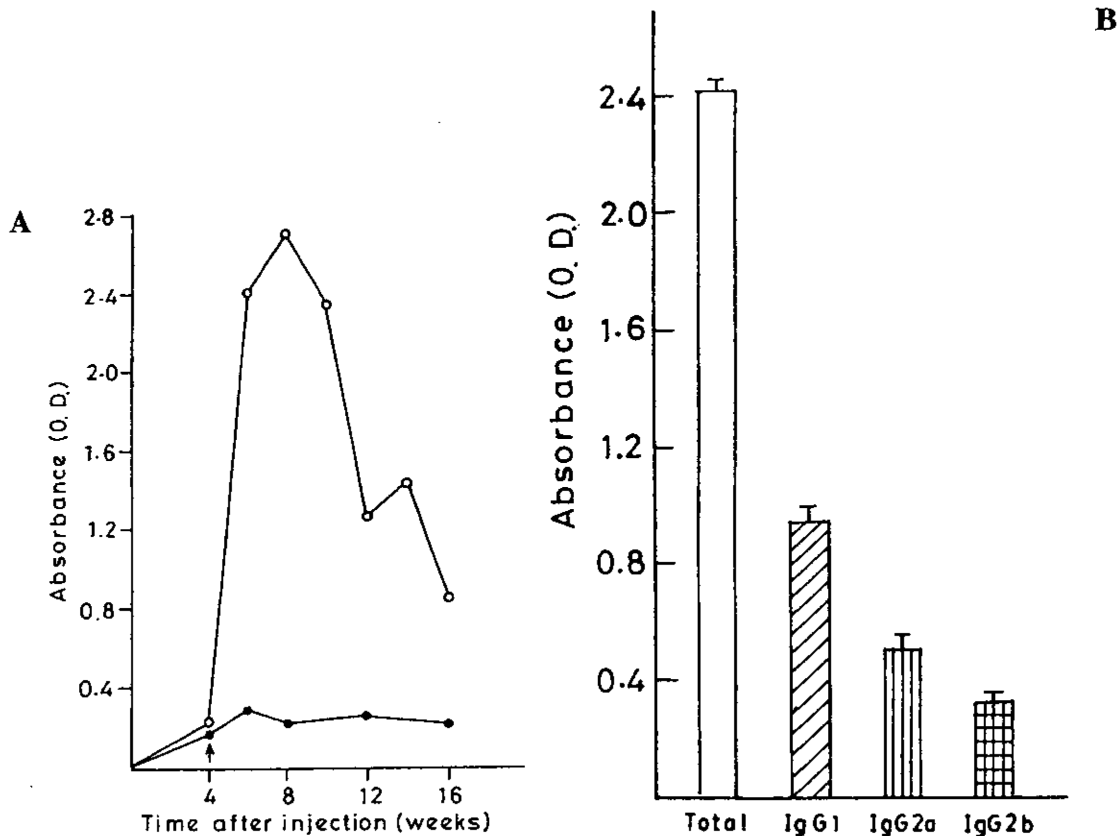

FIG. 3. Effect of coinjection of granulocyte-macrophage colony-stimulating factor (GMCSF) expression plasmid on antibody responses. **A:** Groups of five mice were injected with pcDNA-ORF2 alone (closed circles) and pcDNA-ORF2 and pGM-CSF (open circles). The arrow indicates the time of boost. The anti-ORF2 antibodies were measured in the pooled sera by enzyme immunoassay (EIA). Values shown are a mean of two separate experiments. **B:** Immunoglobulin (IgG) isotype profile in the sera of animals immunized with pcDNA-ORF2 and pGM-CSF. Anti-ORF2 specific total IgG, IgG_1, IgG_{2a}, and IgG_{2b} antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean ± SD for five animals.

4B, the ratio of IgG_1 and IgG_{2a} antibodies was almost equal in the sera suggesting that boosting with protein enhanced both IgG_1 and IgG_{2a} antibodies (Fig. 4B).

DISCUSSION

The remarkable ability of DNA immunization to induce humoral and cytotoxic immune responses against viral, bacterial and parasitic pathogens has led to the widespread application of this technology to vaccine development (11,31). However, the efficacy of different DNA vaccines has varied widely.

In this study, we have shown that mice vaccinated with plasmid DNA expressing the HEV ORF2 protein develop long-lasting antibody responses against the protein. It has previously been shown that immunization with a plasmid encoding the ORF2 gene of the Burmese HEV strain cloned into the eukaryotic expression vector produced long-term humoral immune responses in mice (13). The titers obtained in that study were variable. We show here that coinoculation of plasmids expressing cytokine genes along with HEV ORF2 results in increased antibody responses, which are long lived. We further show that these antibody responses elicited after DNA immunization can be boosted further with protein.

It is well established that cytokines can be used to enhance or redirect immune responses elicited by a

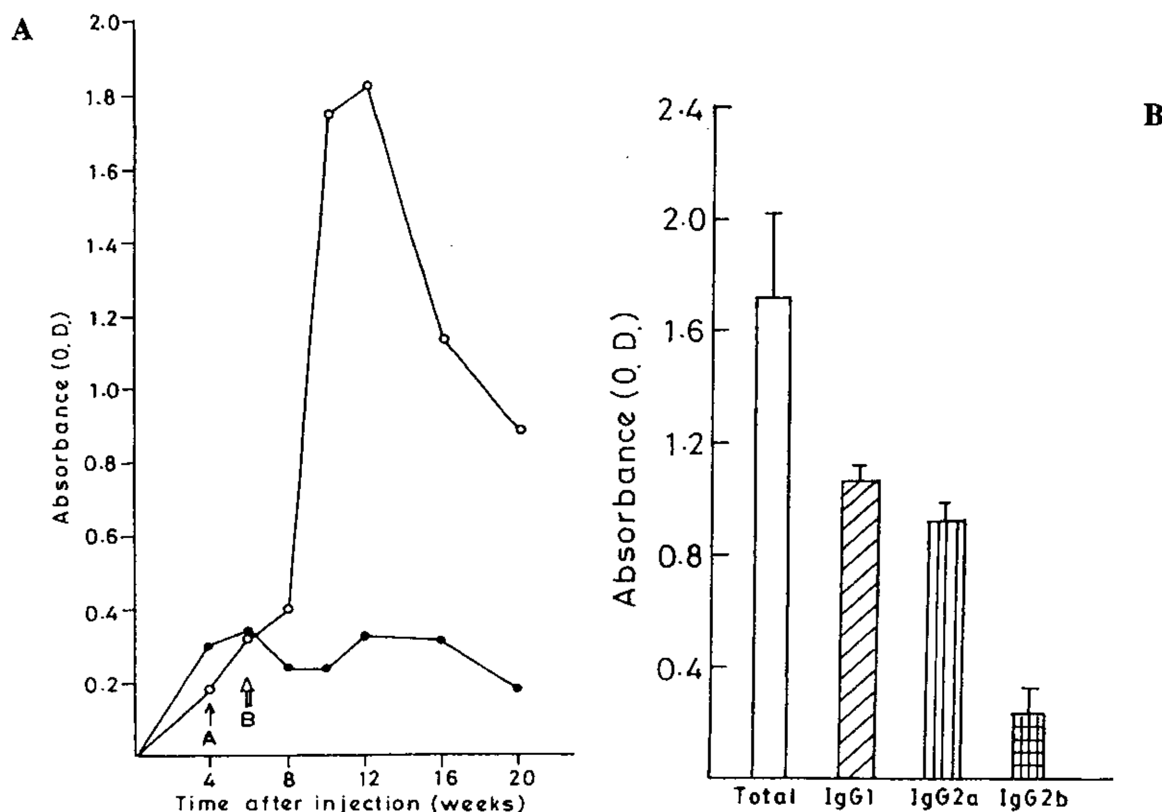

FIG. 4. **A:** Kinetics of appearance of anti-ORF2 antibodies. The figure shows the antibodies in groups of animals immunized with pcDNA-ORF2 alone (closed circles) or in animals primed with pcDNA-ORF2 and boosted with 10 μg of ORF2 VLPs (open circles). Arrow A indicates the time of pcDNA-ORF2 boost to both the groups and arrow B indicates the time of protein boost to one group only. The anti-ORF2 specific immunoglobulin G (IgG) antibodies were detected by enzyme immunoassay (EIA). Four mice in each group were used and the results are a mean of two different experiments. **B:** IgG isotype profile in the sera of animals primed with pcDNA-ORF2 and boosted with VLPs. Anti-ORF2 specific total IgG, IgG_1, IgG_{2a}, and IgG_{2b} antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean ± SD for four animals.