食べる腸管感染ウイルスワクチンの開発
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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、ノーウォークウイルスおよび
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およびジャガイモを作
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クイザルに経口投
与したところ、マウスの同様の抗体産生が誘導された。

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A.　研究目的

　ワクチンは、安価であること、特に子どもの場合注射器による接種ではなく経口投与できること、発熱などの副作用がないこと、コールドチェーンが整備されていない熱帯地域へ常温で供給できること、開発途上国でも自主生産できるワクチンであること、が理想である。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きた疾患である。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ノーウォークウイルス（Norwalk virus、NV）、および開発途上国の経口伝播型急性肝炎の主要病原体であるE型肝炎ウイルス（Hepatitis E virus、 HEV）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。NVはわが国では小型球形ウイルス（small round structured virus、SRSV）とも呼ばれているウイルスである。ノーウォークウイルス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。一方、HEVはわが国へも輸入感染症として近年持ち込まれるケースが多く、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は最近の国際社会、特に日本を含む先進国に対して提言されたCVI（Children'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）ノーウォークウイルス（NV）構造蛋白を産生するトランスジェニック植物の作製

　NVはエンベロープを持たない直径約38nmの小型の球形ウイルスである。ゲノムは約7.6kb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1.6kbのORF2は約530アミノ酸、分子量58kDaの構造蛋白をコードする蛋白である。1987年千葉で分離されたGenogroup 1 に 属 す る Hu/NLV/Chiba407/1987/JP（千葉株）および

Genogroup II に 属 す る Hu/NLV/Chia104/1997/JP（104株）のORF2全領域をカセットベクター pIBT210のSma I 部位にクローン化した。さらにこのプラスミドからカリフラワーモザイクウイルス35S プロモーター、タバコエッチウイルス5'-UTR、目的とする遺伝子、及び3' Soybean vagetative storage protein を含むHind III-Eco RI fragmentをbinary vector pGPTV-Kan に ク ロ ー ン 化 し 、 Disarmed Ti plasmidをもつアグロバクテリアを形質転換した。ジャガイモの若葉にアグロバクテリアを感染させ、定法にしたがってトランスジェニックトマトとジャガイモを作製した。

2）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。

3）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。常法に従っ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 VLPSs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1000×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 VLPSs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）ノーウォークウイルス構造蛋白を発現するトランスジェニック植物の作製

これまでに組換えバキュロウイルスで中空粒子の産生に成功しているNV 9種のうち、Genogroup I の千葉株とGenogroup II の 104株の構造蛋白領域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。千葉株はジャガイモに、104 株 は 既 に Genogroup I に 属 す る Hu/NLV/NV/1968/US（ノーウォーク株）の構造蛋白を発現しているジャガイモとトマトの葉に重感染後、形質転換体を抗生物質で選択した。ウエスタンブロット法で蛋白発現をみたところ千葉株では約30のクローンで発現が確認できた。一方、104株では0.2-0.35ng/ugの発現量を持つトマトを5ヶ得た。ノーウォーク株の発現を確認中である。

2）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。

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

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。血中IgG抗体の上昇、あるいは腸管IgA抗体の検出をみて、チャレンジを行い感染防御あるいは発症阻止の有無を観察する予定である。

D. 考察

　本年度はNV構造蛋白を産生するトランスジェニックトマトとジャガイモが得られた。ジャガイモではGenogroup ⅠとGenogroup Ⅱに属し血清型が異なる2種類の中空粒子をひとつの植物体で産生することを試みた。先に米国で行われたタバコを用いた同種の実験では、組換えバキュロウイルスで産生されるVLPs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おり、今回用いたトマトやジャガイモでも粒子の産生が十分期待できる。またひとつの植物体で2種類のVLPsを発現する試みは全く初めてのことであり結果が待たれる。これらはE型肝炎ウイルスVLPs同様、経口投与実験で腸管粘膜に抗体産生を誘導出来ることが十分期待でき、食べるワクチンとして有望である。

E. 結論

NV構造蛋白を産生するトランスジェニックトマトとジャガイモが得られた。ジャガイモではGenogroup ⅠとGenogroup Ⅱに属し血清型が異なる2種類の中空粒子をひとつの植物体で産生することを試みた。またHEV構造蛋白を発現するトランスジェニックトマトが作製できた。組換えバキュロウイルスで発現したHEV VLPsを用い腸管免疫誘導能をカニクイザルで試験した結果、マウスに比べて低い抗体価ではあったが同様の免疫反応を誘導した。

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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, non-B hepatitis, now called hepatitis E (19). It is transmitted primarily by the fecal-oral route, 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 non-endemic areas have been reported among travelers returning from high HEV-endemic areas. Outbreaks of hepatitis E have occurred 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. Though protracted viremia upto 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 case of co-infection with other hepatitis viruses' (1) where fulminant liver disease leading to high rates of mortality have been reported.

Hepatitis E virus is a spherical, non-enveloped 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 degree of nucleotide and amino acid sequence conservation and includes three open reading frames (ORFs) (28). The N-terminal ORF1 of about 5 kb is predicted to code for the putative nonstructural proteins and the C-terminal region of about 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, besides 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 immunomodulatory 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 EcoRV 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 EcoR1 (to delete the pS2-S sequence) and religated to produce the pIL-2 expression vector. The GM-CSF clone expressing mouse granulocyte-macrophage-colony-stimulating factor (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 pre-immune 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 retroorbital 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 non-fat milk in phosphate buffered saline (PBS) for 1 h at 37ºC. 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 h at 37ºC. Same dilution of the pre-immune 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 pre-immune sera with a cut-off value of 0.05.

For detecting isotype breakdown of the IgG antibody response, the reaction with test serum was followed by incubation at 37°C for 1 h 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 Company.

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 post-immunization were of 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). Though 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 further 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 co-expression on the anti-ORF2 response. An 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 (A, B and C) were inoculated with 100 µg of pcDNA-ORF2. Group B was co-injected 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 following 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 i.e. 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 i.e. pcDNA-ORF2 and pIL2 in group B and C, since boosting with pIL2 alone had no significant effect on the antibody titers. For four weeks after the first injection, the antibody levels in all three groups were similar. However, after six weeks of the first injection (i.e. two 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 upto 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 plasmid 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 post-immunization were determined for animals in Group C. As shown in figure 2B, the ratio of IgG1 and IgG2a antibodies was almost equal, suggesting that co-administration of an IL-2 expression plasmid enhanced both IgG1 and IgG2a 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 co-expression 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 co-injected with pcDNA-ORF2. The control group received only plasmid pcDNA-ORF2. The sera were analyzed after four weeks of injection and antibodies to pORF2 were detectable in both the groups. At this time the two groups were boosted with the respective plasmids and the sera analyzed every two weeks thereafter for upto sixteen weeks (Fig. 3A). After two 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 i.e. pcDNA-ORF2 and pGM-CSF, the increase in antibody responses was observed. The effect of co-injection of GM-CSF though very pronounced was short lived, decreasing substantially 10-16 weeks post-immunization (Fig. 3A).

The anti-ORF2 IgG isotypes in the immune antiserum collected at 8 weeks post-immunization were measured in mice co-inoculated with GM-CSF. As shown in figure 3B, although the response was of a mixed type, there were more anti-ORF2 antibodies of the IgG1 isotype compared to the IgG2a isotype.

Boosting of humoral response. As shown earlier, boosting with DNA four weeks after the primary immunization resulted in a slight increase in antibody titers (Fig. 1B). It has been shown in other studies that 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. It was observed that the titers remained almost unchanged for two weeks after the boost with protein but after four weeks there was a sharp increase in these titers (Fig. 4A). The titers reached peak levels after twelve weeks of the initial inoculation followed by a sharp decline. The antibody levels were about half of the peak level at sixteen weeks and decreased slowly after this time (Fig. 4A). In a control group of ten animals, each immunized with only 10 μg of the ORF2 VLPs (in saline), the antibody response was weak and was observed for only six weeks. The optical density mean values after four 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 post-immunization were determined for animals primed with pcDNA-ORF2 and boosted with VLPs. As shown in figure 4B, the ratio of IgG1 and IgG2a antibodies was almost equal in the sera suggesting that boosting with protein enhanced both IgG1 and IgG2a 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 co-inoculation 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 DNA vaccine, but the level of enhancement varies widely with different vaccines (3,7,12). IL-2 has previously been characterized as a factor that augments specific immune responses and has been shown to be an effective adjuvant for subunit and inactivated virus vaccines (23,34). While in most studies the cytokine expression plasmids have been used, in some cases soluble protein also has been used (7). The effect of administration of soluble protein was not very significant because of brief circulatory half-life as compared to the protein produced *in vivo* by the expression plasmid (7). We therefore used cytokine expression plasmids in conjunction with a plasmid expressing HEV ORF2 for immunization of mice. It was observed that co-expression of IL-2 increased the anti-ORF2 titers several folds and these were maintained for longer times. The IL-2 expression *in vivo* may be the reason for increase in antibody responses since IL-2 directly activates macrophage functions and also stimulates the release of other secondary mediators such as GM-CSF, which may also contribute to macrophage activation (25). The days immediately following the priming of the immune response appear to be an optimal window for augmentation of the immune response. Since it was observed that IL-2 administered at day 4 after ORF2 plasmid

inoculation provided maximum stimulation, it appears necessary to first prime the immune system with DNA encoding the antigen and then amplify the response with this cytokine.

Granulocyte-macrophage colony stimulating factor (GM-CSF) has been reported to enhance antibody response (10) and plasmids expressing GM-CSF has been used previously for this purpose. Co-inoculation of a plasmid expressing GM-CSF with a rabies virus DNA vaccine increased the rabies-specific antibody responses in mice (35). It has been shown that co-administration of GM-CSF and HIV-1 expression plasmids enhanced both Th1 and Th2 type responses (24). For a hepatitis B vaccine also both responses were affected by co-expression of GM-CSF (6). Using DNA encoding the hepatitis C virus core protein, it has been reported that cellular and humoral responses were enhanced by GM-CSF expressing plasmids (12). This cytokine may substantially enhance the ability of the host to respond to viral structural proteins by expanding the numbers of antigen-presenting cells as well as augmenting the antigen presenting ability of mature macrophages. In the present study we show that co-expression of GM-CSF resulted in an amplification of the antibody responses to the HEV ORF2 protein significantly. This favored the generation of Th2 cells as evidenced by an increase in the IgG1 isotype fraction.

The antibody responses generated after DNA immunization can be augmented by administration of the antigenic protein as shown in the case of hepatitis B surface antigen (8,9). The data presented here using hepatitis E virus ORF2 gene also are in agreement with those findings. In the present study a lag period was observed between the protein boost and the stimulation of immune response. Since DNA immunization relies on low numbers of transfected, antigen expressing cells to raise immune responses, it possibly generates fewer memory cells that require time for activation and clonal expansion. Although similar antibody titers were obtained on immunization with protein only, the amounts of protein required were at least five fold higher than the amounts required to boost the response after priming with DNA.

With a DNA vaccine for hepatitis B it has been shown that a recombinant protein boost given to a DNA primed chimpanzee induced a rapid and strong elevation of anti-hepatitis B antibody titers (9). Further in rhesus macaques immunized with HIV/HBsAg fusion proteins, injection of a HBsAg expressing plasmid three years later was immediately followed by a rise in anti-HBs titers (5). These studies suggest that a combination of DNA priming/protein boosting can be followed to induce strong and broad-based antibody responses.

The present studies show that the HEV ORF2 gene can be used to generate long lasting and elevated immune responses in the murine model when used in combination with cytokines. Cytotoxic immune responses are also important in clearing viral infections through virus-specific $CD8^+$ T cells (CTLs) which recognize and kill virally infected cells. The ability of HEV ORF2 DNA vaccination to generate virus-specific CTLs in addition to a humoral response, and the evaluation of these approaches in a rhesus monkey model of HEV infection remain to be carried out in future.

ACKNOWLEDGEMENTS

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