とする情報の解析を十分に行い、希望する結果・内容の提供用件を満たす手段として適当であるかに対する見解と、計画しているリスクアセスメントによって求めている結果に対して、現況で想定できる提言および、それが実際の施策にどのように反映しえるかについての検討：

食中毒統計から、カキの生食と不十分な加熱調理での摂取が、原因とする食中毒事件が明らかになっている。食中毒事例のうちノロウイルスによるものが大きな割合を占めていることは明らかである。

これによるリスクは、現在までに解っている基礎実験データより、ウォッシュ・アウト期間に何らかの基準を設けることにより減少できる可能性が示唆されている。しかし、その効果は完全なものとは言えない。

現在のところノロウイルスによる下痢症発生のリスクの大きさは定量的に明確に示されておらず、検出技術の向上によってノロウイルスが報告される様になったこともあり、一般の関心も高まり、生産者、消費者双方から新しいカキの安全性の規格基準の設定の希望が出てきている。微生物学的リスクアセスメントの結果からリスクの大きさの程度、微生物学的新基準、生食用海産物の養殖や取り扱いに関するガイドラインおよび患者数減少のための対策と食品以外の原因によるノロウイルス患者の実態把握の方法などへ対する示唆、提言が期待できる。また、下水処理場におけるウイルス除去効率を上げる効果についても科学的に推定ができる。

しかし、カキ汚染は乳幼児から高齢者のノロウイルス排泄状況、下水処理場でのノロウイルス除去状況、降雨量と海域への流入量、海域での海流、養殖海域での筏の河川水の受ける影響等を総合的に研究しなければ実態の究明は困難である。

＊ 仮にリスクアセスメントが必要であることが確認されたとして、マネージャー側からアセッサーへ問いかける初期の質問事項及び解析を希望する事項：

ノロウイルスによる真の年間罹患者数および、集団発生における感染経路と原因の内わけが現行のシステムで十分に把握されているか？

上に挙げたマネジメントオプションの効果と効率の比較。

1） カキの十分な加熱調理の指導
2） 養殖海域、養殖過程の産物、出荷時の産物の微生物学的基準の変更および強化
3） UV 殺菌水等による出荷前の洗浄、さらに効果的は洗浄法の確立
4） 出荷前にウォッシュ・アウト期間を設けることの有効性
5） 下水処理場におけるウイルス除去効率を上げることの有効性
6） 現在の入手可能な情報と、不足している知見および情報

＊ この病原体・媒介食品の組み合わせに対する、既存の国家単位のリスクアセスメントの存在：

- 欧州共同体より国際的リスクアセスメントの枠組みに従ったリスクの検討報告がだされている (European Commission, 2002)。

・ New Zealand Food Safety Authority による Norwalk-Like Virus in Mollusca (Raw)
http://www.nzfsa.govt.nz/science-techno...es/norwalk-like-virus-in-raw-mollusca.pdf

* リスクアセスメントを実行することも含め、リスクマネジメント活動を促進するその他の関連した科学的知見やデータの存在：

最近の知見によれば、カキおよび養殖の二枚貝に関しては、公衆のリスクを減少し得る、生産者側とも合意しあえる「ワッシュ・アウト」期間を提示できるものと考えられる。

* Codex に準じた、リスクマネジメントのガイダンスを作成するのに役立つ情報源（研究機関、官製情報、個人研究者など）と科学者：

厚生労働省----------食中毒統計、食中毒詳報、感染症発生動向調査、病原微生物検出情報
国立感染症研究所----------ウイルス第2部（武田）、感染症情報センター（岡部、西尾）
東京都立衛生研究所----------（関根）
国立医薬品食品衛生研究所--------食品衛生管理部（山本、春日、鈴木）
東京大学工学系研究科----------都市工学専攻（片山）
海外---------- David Vose, Greg Paoli
"Opinion on veterinary measures relating to public health on Norwalk-like viruses", adopted on 30-31 January 2002 by European Commission, Health & Consumer Protection Directorate-General.

* リスクマネジメントを行う上で障害となり得る情報の欠如の存在領域：

1）カキにおける感染を有するノロウイルス（現在組織培養が出来ないので感染性の有無は知る手段が無い）の定量の手段を有しないので、代用できる方法の確立が望まれる。
2）確立した、高感度の定量的ウイルス同定システムの構築
3）養殖条件（温度、期間、海域内配置、プランクトン発生等）の記載形式が統一されておらず、記録が不定期
4）集団発生の際の原因食材のトレースバックのシステムが不完全（バッチ、ロットの記載が義務化されていない、収穫時期の記載義務が不十分、養殖海域のどの部分からの収穫か記録がない等）
5）臨床症状の発生に必要なウイルス量が不明である。このウイルスに関するD-Rカーブがほとんど存在しない
6）ノロウイルスに関する人免疫の情報が少ない（ハイリスク・グループの存在の有無も含めて不明）。
7）加熱調理、調理手法、消毒などのノロウイルスに対する効果の情報不足

8）サーベイランスからのノロウイルス患者情報の不足（現行の感染症サーベイランスでは感染性胃腸炎の中に含まれて報告されるため、実数は不明）

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Age-specific antibody to hepatitis E virus has remained constant during the past 20 years in Japan

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SUMMARY. We investigated the presence of antibodies to hepatitis E virus (anti-HEV) and hepatitis A virus (anti-HAV) by enzyme immunoassays in sera from 1015 individuals collected in 1974, 1984 and 1994. Age-specific profiles of anti-HEV remained unchanged with a peak at 40–49 years, while those of anti-HAV started to increase in individuals aged 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. These results suggest that a silent HEV infection has been taking place in the last 20 years or so in Japan, while HAV infection has been terminated at least since 1974.

Keywords: hepatitis A virus, hepatitis A virus antibody, hepatitis E virus, hepatitis E virus antibody, seroepidemiology.

INTRODUCTION

Hepatitis E virus (HEV) is transmitted mainly by the faecal–oral route, and causes waterborne outbreaks and sporadic cases of acute hepatitis in developing countries with poor sanitary conditions [1]. Outbreaks of HEV have been primarily noted in developing countries, whereas cases in developed countries were considered to have been exposed in foreign countries. However, cases of acute hepatitis because of indigenous HEV strains were reported in patients in the United States, Europe and Japan who had never travelled abroad [2–4]. Recently, HEV strains have been isolated from pigs in developed countries, which are closely related to local human HEV strains, suggesting zoonotic infection [5–7]. Because of these lines of evidence, HEV has attracted increasing attention even in developed countries where HEV is not endemic. For example, in Japan, more than 20% of acute, sporadic hepatitis cases are non-A, non-B, non-C [8].

The impact of HEV infection in developed countries, however, has not been fully explored. Past exposure to HEV can be examined by enzyme-linked immunosorbent assay (ELISA) by detecting antibody to HEV (anti-HEV). In the present study, we surveyed the extent and changes of HEV infection in Japan by testing sex- and age-specific prevalence of anti-HEV in serum samples collected in 1974, 1984 and 1994. Using the same samples, we also tested for antibodies to hepatitis A virus (anti-HAV) for comparison.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAV, hepatitis A virus; HEV, hepatitis E virus; VLP, virus-like particles.

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MATERIALS AND METHODS

Serum samples

A total of 1015 samples were selected at random from the Serum Reference Bank of the National Institute of Infectious Diseases, Tokyo. They were obtained from healthy volunteers aged from 0 to 89 years (median 35.6 years) living in seven prefectures in the central part of Japan. Of them, 349 were collected in 1974, 324 in 1984 and 342 in 1994. The present study, was reviewed by the ethical committee of the National Institute of Infectious Diseases.

Hepatitis viral markers

Anti-HAV (total antibody) was determined by radioimmunoassay using a commercial kit (HAV-AB RIA kit; Dainabot Co., Ltd, Tokyo, Japan). Positive and negative results were judged according to the manufacturer's instructions with intermediate results recorded as negative. Anti-HEV was determined by ELISA using the method of Li *et al.* [9]. Briefly, wells of microtitre plates were coated with purified virus-like particles (VLP) of HEV expressed by a recombinant baculovirus. One hundred microlitres of test serum was then added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed six times with 10 mM phosphate

buffered saline containing 0.05% Tween 20, and then 100 μL of buffer containing goat anti-human IgG conjugated with horseradish peroxidase was delivered to each well. The plate was incubated for a further 1-h period at 37 °C, washed six times, and thereafter, each well received 100 μL of buffer containing orthophenylenediamine. The plate was incubated at room temperature for 30 min, and then 50 μL of 4 N H_2SO_4 are added to each well. The absorbance at 492 nm was recorded and positive and negative results were scored as described by Li *et al.* [9].

Statistical analyses

Statistical analyses were performed using the chi-square test, and $P < 0.05$ was considered significant.

RESULTS

Age-specific prevalence of anti-HEV and anti-HAV

Basic patterns of age-specific prevalence of anti-HEV were similar in the three examination years (Fig. 1). The prevalence of anti-HEV increased gradually until 40–49 years old, and then decreased slowly with age. In contrast, the prevalence of anti-HAV was almost nil in people younger than 20 years, increased steeply at a certain age range, and then reached 80–90% in older people in all of the three years of examination. The age range at which the prevalence of anti-HAV started to increase sharply was 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. Thus, it shifted by 10 years at each examination year. Anti-HAV was significantly more prevalent than anti-HEV in all age ranges over 30 years in 1974 ($P < 0.001$ in all). Similarly, anti-HAV was significantly more prevalent in all age ranges over 40 years in 1984 ($P < 0.001$ in all), and in all age ranges over 50 years in 1994 ($P < 0.001$ in all).

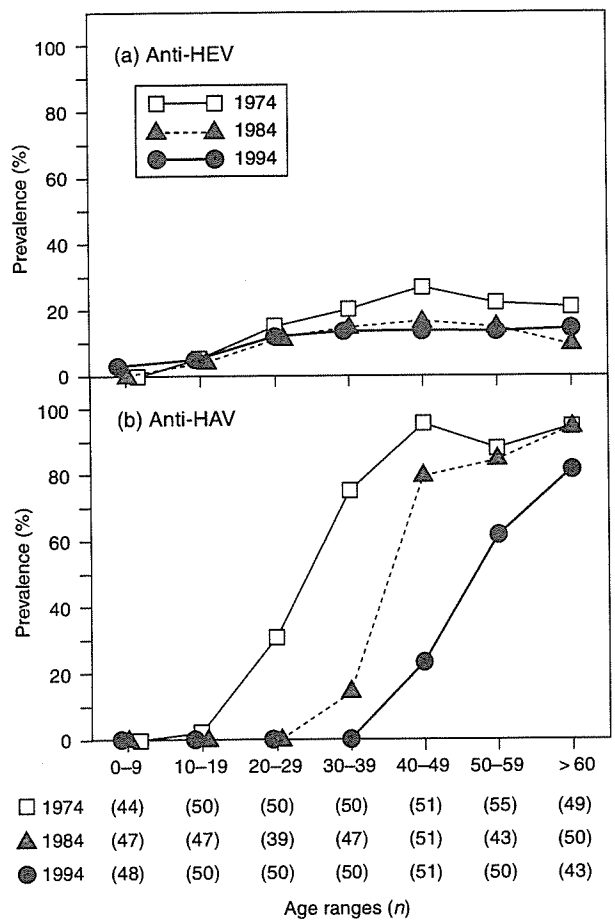

Fig. 1 Age-specific prevalence of anti-HEV and anti-HAV in Japan at three different times. Number of individuals tested in each age group and year is indicated below in parentheses.

Sex- and age-specific prevalence of anti-HEV

Figure 2 illustrates the prevalence of anti-HEV in serum samples from different age groups of healthy Japanese volunteers collected in 1974, 1984 and 1994 stratified by

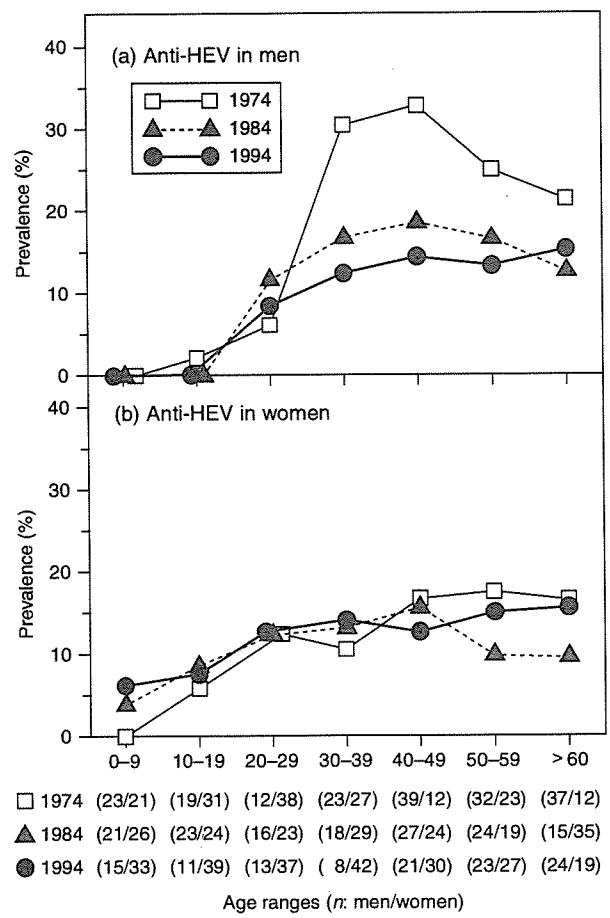

Fig. 2 Sex and age-specific prevalence of anti-HEV in Japan at three different times. Numbers of men/women tested in each age group and year are indicated below in parentheses.

sex and age. Although basic profiles of the prevalence of anti-HEV did not differ between men and women, anti-HEV in men was significantly more frequent in 1974 (21.6%) than in 1984 (11.1%, $P = 0.012$) and 1994 (10.4%, $P = 0.013$); the difference is attributed to a high frequency of anti-HEV in age groups older than 30 years in 1974. In sharp contrast, the age-specific prevalence of anti-HEV in women stayed unchanged in the three different years examined (11.0% in 1974, 10.6% in 1984 and 11.9% in 1994). Differences in the prevalence of anti-HEV between men and women were significant only in the year 1974 ($P = 0.008$). Age-specific prevalence of anti-HAV was quite similar between males and females in each year of examination (data not shown).

DISCUSSION

Many immunological methods have been developed for the determination of anti-HEV utilizing natural and recombinant viral proteins as antigens. They are, however, disappointingly nonspecific and have been shown to yield discrepant results using the same panel of sera with or without anti-HEV [10]. Lack of reliable serological assays for the detection of anti-HEV has hampered the accurate examination of exposure to HEV in various epidemiological and clinical settings.

Recently, Li *et al.* [11] succeeded in developing VLP using a recombinant baculovirus containing the coding sequences for capsid protein of the virus. The VLP appear to have similar antigenicity to the authentic HEV particles [12]. Using these VLP, a novel ELISA for anti-HEV has been developed that is sensitive and specific in seroepidemiological surveys for HEV infection. A considerable proportion of Japanese adults (around 10–20%) appear to have had previous exposure to HEV, although Japan is not endemic for hepatitis E [9,13].

The clinical features of HAV infection are similar to those of HEV infection, in that they both are transmitted by the faecal–oral route and cause acute hepatitis without chronic sequelae. In the present study, serological markers of HAV and HEV infection were determined and compared among healthy Japanese volunteers at three different time points (1974, 1984 and 1994). Age-specific prevalence of anti-HAV increased steeply and reached 90% at a certain age range dependent on the year of examination. The age range at which anti-HAV increased shifted by 10 years in subsequent time points, indicating that HAV infection was endemic several decades ago in Japan and has been contained thereafter. This would be mainly because of an improvement of sanitary conditions in Japan since the 1950s.

Age-specific profiles distinct between anti-HAV and anti-HEV during the last 20 years in Japan would be not only of epidemiological but also of clinical relevance. The prevalence of anti-HAV increasing with age involving by far the most aged individuals signifies a life-long immunoprotection against HAV. By sharp contrast, the prevalence of anti-HEV did not increase linearly with age, and peaked in individuals aged 40–49 years. Furthermore, unlike anti-HAV that has become increasingly absent in younger age groups, anti-HEV was detected in younger individuals aged 20–29 years in both men and women, and among women aged <20 years, in the three examination years. Similar age-specific profiles of anti-HEV have been reported in India [14]. Prevalence of anti-HEV in Japanese individuals younger than 30 years old was somewhat higher in the present study, than the almost zero prevalence reported by Li *et al.* [9]. Although there was some difference in percentages, the basic pattern of age-specific prevalence of anti-HEV was almost similar between the two studies.

Based on the age-specific distribution of anti-HEV, the exposure to HEV has not decreased during the past 20 years in Japan, unlike that to HAV. This implies that the principal transmission route of HEV may be different from that of HAV and would not be prevented only by improved sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [14]. Zoonotic transmission of HEV through domestic and wild animals may account for the perpetuation of HEV infection, and deserves to be examined in future studies. Recently, transmission of HEV from pigs and deer to human beings has been reported in Japan [15,16].

In conclusion, exposure to HEV has stayed unchanged during the last 20 years in Japan, contrasting with HAV, which has diminished over the same period. These results warrant closer attention to infection with HEV, especially because it can induce fulminant hepatitis not only in pregnant women in developing countries [14], but also in sporadic cases in developed countries [15].

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Challenges in creating a vaccine to prevent hepatitis E

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Summary

Recombinant hepatitis E virus capsid protein (HEV CP) assembles orally immunogenic virus-like particles (VLP) when expressed in an insect cell system. We used plant expression cassettes, pHEV101 and pHEV110, for transformation of potato to express HEV CP, and 10 independent transgenic lines of HEV101 and 6 lines of HEV110 were obtained. ELISA for HEV CP was performed on tuber extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber depending on the transgenic plant line. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. Although Western blot showed that apparently intact HEV CP accumulated, we observed very limited assembly of virus-like particles in potato tubers. Oral immunization of mice with transgenic potatoes failed to elicit detectable anti-CP antibody response in serum, suggesting that VLP assembly is a key factor in orally delivered HEV CP vaccines.

Keywords: Hepatitis E; HEV; VLP; Transgenic potato

1. Introduction

Hepatitis E virus (HEV) is a causative agent of hepatitis E that occurs in many developing countries [1], and this virus is currently classified into a tentative genus, "Hepatitis E-like viruses." HEV is transmitted mainly by the fecal-oral route, and large epidemics due to this virus are often associated with contaminated water [2,3]. Hepatitis E has been formerly known as an enterically transmitted non-A, non-B hepatitis [4]. The fact that HEV can survive in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions.

HEV contains a single-stranded positive-sense approximately 7.5 kb RNA molecule that is 3′ polyadenylated and includes three open reading frames (ORFs). ORF1, mapped in the 5′ half of the genome, is thought to encode viral nonstructural proteins. ORF2, located at the 3′-terminus of the genome, encodes a 72 kDa protein for the putative viral capsid. ORF3, with unknown function, is mapped between ORF1 and ORF2 [5]. In the absence of an appropriate cell culture for HEV propagation, research has focused on the expression of the ORF2 protein in heterologous systems. Recently, virus-like particles (VLP) of recombinant hepatitis E virus (rHEV) were produced by using a baculovirus system carrying an N-terminally truncated *ORF2* gene of the Burma strain [6]. Thus, rHEV VLP were formed in Tn5 cells and could be collected from the culture supernatant.

In order to evaluate the potential of rHEV VLP as an oral immunogen, we analyzed the immune responses in mice and monkeys after oral administration [7,8]. The animals were orally inoculated with purified rHEV VLP without adjuvant. ELISA indicated that oral immunization with rHEV VLP induced immune responses in both mice and monkeys. In addition, the monkeys were completely protected from infection when challenge was carried out with native HEV, suggesting that rHEV VLPs are a potential mucosal vaccine for HEV infection.

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For expression and delivery of recombinant subunit vaccine antigens including Norwalk virus capsid protein (NVCP), transgenic plants have been created [9]. Clinical trials using potatoes expressing NVCP 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 [10]. Previous studies with NVCP expression in tobacco and potato cells demonstrated that subunits assembled to form 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 [9]. 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.

In this study, we expanded our effort to create transgenic plants that express HEV capsid proteins (HEV CP).

2. Materials and methods

2.1. Preparation and purification of rHEV VLP

The molecular cloning and construction of a recombinant baculovirus Ac5480/7126 harboring the HEV capsid protein gene lacking 111 amino acids at the N-terminal were described previously [6]. The rHEV VLP were prepared using Tn5 cells infected with Ac5480/7126 at a multiplicity of infection of 10. Following 7 days of incubation at 26.5 °C, intact cells and cell debris were removed from the culture medium, and the rHEV VLP were concentrated by centrifugation and purified by isopycnic binding in CsCl gradient. A visible band containing rHEV VLP was collected, and the rHEV VLP were diluted and pelleted by centrifugation.

2.2. Western blot assay

Leaf samples were extracted by FastPrep (speed 5, 30 s) in 4 ml/g leaf of 50 mM sodium phosphate pH 6.6, 50 mM NaCl, 50 mM sodium ascorbate, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, and clarified for 2 min at 4 °C in microcentrifuge. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV (1:1000), visualized with ECL+ (anti-guinea pig IgG-HRP 1:5000) on STORM scanner.

2.3. Antigen-capture ELISA

Potato leaf or tuber extracts were prepared as described above in Section 2.2. Microtiter plates were coated with 50 μl per well of rabbit anti-HEV serum diluted 1:10,000 in carbonate/bicarbonate coating buffer overnight at 4 °C. Insect cell-derived HEV VLP reference standard was diluted in PBST/1% dry milk at 100 ng/ml and two-fold dilutions down to 3.125 ng/ml. Leaf or tuber extracts were diluted 25- and 50-fold in PBST/1% dry milk. The reference standards and plant extracts were loaded at 50 μl per well and incubated at 37 °C for 1 h. Wells were washed with PBST and then probed with guinea pig anti-HEV serum diluted 1:5000, followed by goat anti-guinea pig IgG-HRP conjugate (Sigma) diluted 1:5000. Color was developed using TMB substrate solution for 5 min.

2.4. Construction of plant expression vector

Intermediate plant expression cassettes were constructed using a vector pIBT210 [11]. Since two truncated forms

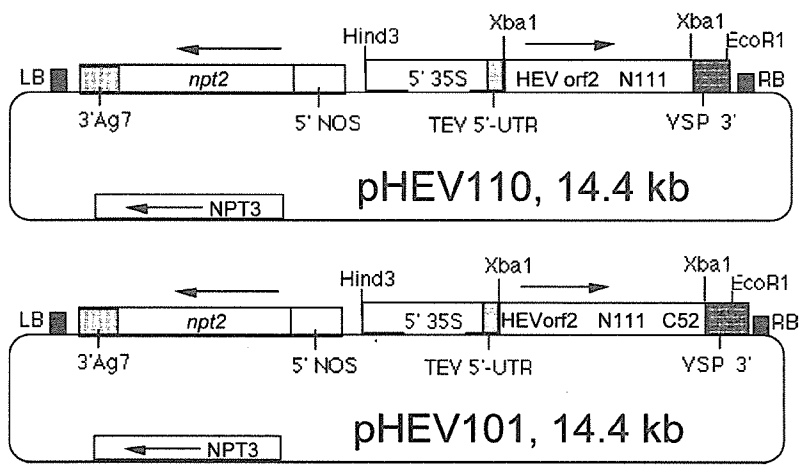

Fig. 1. Plant transformation vectors for expression of HEV CP. Binary T-DNA plasmid vectors, pHEV101 and pHEV110, for expression of HEV CP, p54 and p58, are shown. They contain left border (LB) and right border (RB) sequence motifs that delineate the DNA to be transferred (T-DNA) and integrated into nuclear chromosomal DNA. Within the T-DNA borders lies a selectable marker (*ntp2*), which confers resistance to the antibiotic kanamycin, and will allow specific regeneration of transformed plants. Also included are expression cassettes for HEV CP, which are driven by the constitutive CaMV 35S promoter linked to the tobacco etch virus (TEV) 5′-UTR, which acts as a translational enhancer, and terminated by the soybean VSP 3′ end [9].

of HEV ORF2 appeared to yield VLP assembly in insect cells (ΔN111 and ΔN111/ΔC52), we inserted these into pIBT210. Then, the expression cassettes were transferred into a binary vector (pGPTV-Kan) for use in *Agrobacterium*-mediated delivery of foreign DNA into plant cells. These constructs, pHEV101 (ΔN111/ΔC52) and pHEV110 (ΔN111), are shown in Fig. 1.

3. Results

3.1. Characterization of rHEV

The capsid proteins of HEV with its N-terminal 111 amino acids truncated were expressed with a recombinant baculovirus in insect cells, where the capsid proteins self-assembled into VLP [6]. The rHEV VLP were purified by centrifugation and characterized by SDS-PAGE and Western blot assay, where a major protein band with a molecular weight of 54 kDa was observed. The particles possess antigenicity similar to that of authentic HEV particles and consequently they appear to be a good antigen for the sensitive detection of HEV-specific IgG and IgM antibodies [12]. Furthermore, the VLP may be the most promising candidate for an HEV vaccine owing to its potent immunogenicity [7,8]. Therefore, we used the same construct to express HEV CP in the transgenic plant.

3.2. Coding sequence analysis

We first examined the coding sequence for the HEV CP to determine whether the nucleotide sequence should be altered for optimization of plant expression. Codon use is fairly favorable to both dicot and monocot plants. Of 660 total codons, 3.6% are monocot-unfavorable and 12.8% are dicot-unfavorable, defined as either making up less than 10% of codon choice for that amino acid or less than one third the frequency of the most popular codon for that amino acid, inclusive.

3.3. Expression in potato plants

We used pHEV101 and pHEV110 for transformation of potato "Desiree" as described [9,13]. After regeneration of multiple independent kanamycin-resistant lines, we screened leaf samples by ELISA for HEV CP expression. Expression levels ranged up to approximately 0.33% total soluble protein, which is similar to the levels we obtained for NVCP [9]. There was no apparent difference in expression from either construct pHEV101 (ΔN111/ΔC52 coding sequence) and pHEV110 (ΔN111 coding sequence) as the range and maximal expression were similar for both.

We selected the best lines for transplant to the greenhouse and after 2 months growth we assayed leaves for expression of HEV CP by ELISA. We observed that the antigen

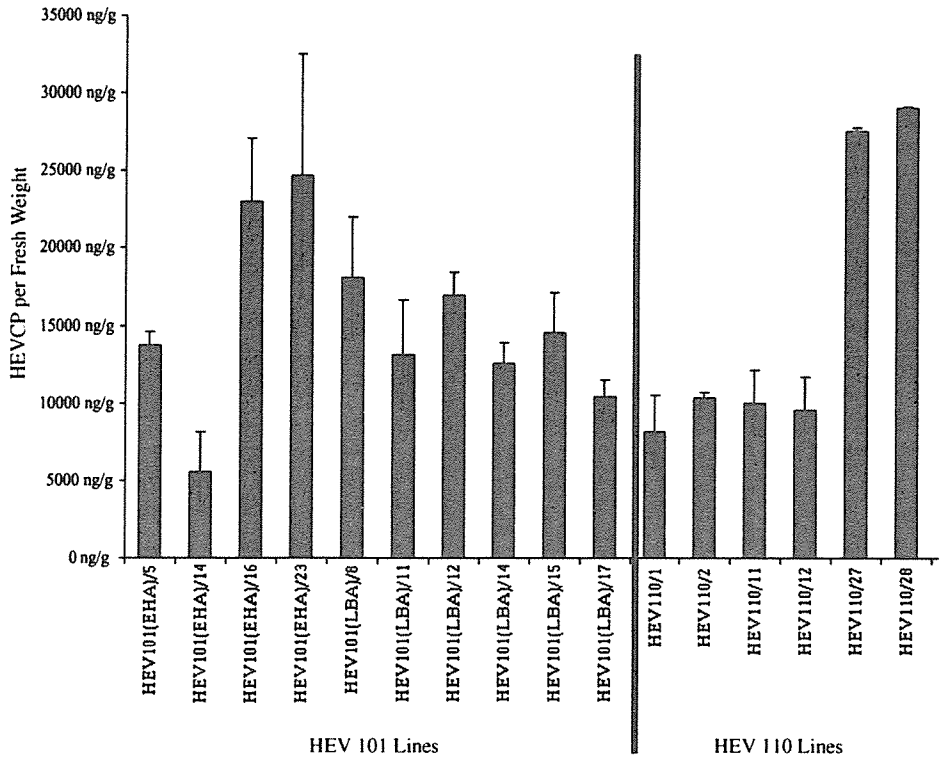

Fig. 2. Expression of HEV CP in tubers of transgenic potato lines. HEV101 (10 lines) or HEV110 (6 lines) tubers were extracted and assayed by ELISA for HEV CP. Error bars indicate standard error for three different tubers from the same line.

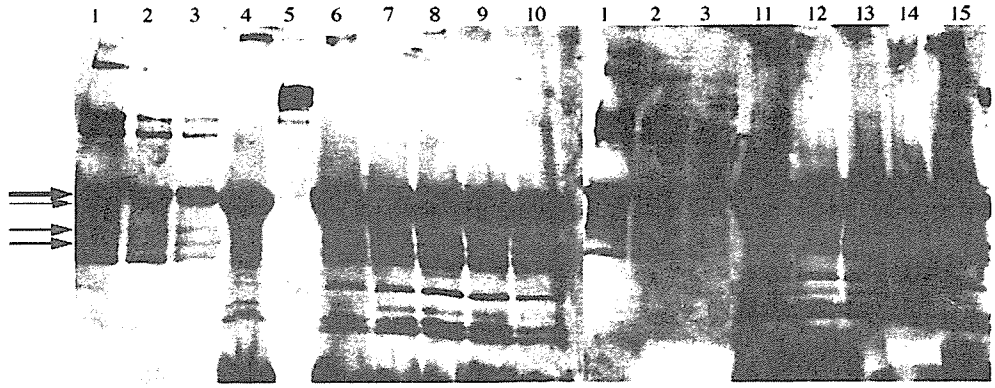

Fig. 3. Western blot of HEV CP expressed in potato leaves. Leaf samples were extracted by FastPrep and clarified by centrifugation. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV, visualized with ECL+ on STORM scanner. Lanes are: (1) 50 ng HEV VLP from baculovirus-infected insect cells, (2) 25 ng HEV VLP, (3) 12.5 ng HEV VLP, (4) untransformed Desirée extract, (5) BioRad Kaleidoscope Molecular Weight Marker, (6) HEV101(EHA)/5, (7) HEV101(EHA)/12, (8) HEV101(EHA)/16, (9) HEV101(EHA)/17, (10) HEV101(LBA)/11, (11) HEV101(LBA)/14, (12) HEV110/1, (13) HEV110/2, (14) HEV110/11 and (15) HEV110/12. Bold arrow indicates major band of VLP sample that corresponds to the 54 kDa HEV CP; thin arrows indicate products of proteolysis.

expression on a total protein basis was reduced four- to five-fold compared to that in tissue-cultured plantlets. This is not unexpected, since soil-grown plants in natural light have higher levels of total leaf protein. Further, it is possible that the recombinant antigen is less stable in the soil-grown plants.

3.4. HEV CP expression in potato tubers

Ten independent transgenic lines of HEV101 and six lines of HEV110 were grown to maturity in the greenhouse and tubers were harvested and washed. ELISA for HEV CP was performed on tuber extracts as described for leaf extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber, depending on the transgenic plant line (Fig. 2). This compares well with the expression of NVCP in potato tubers [9] and is better than expression of *E. coli* LT-B protein in potato [13]. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. We used these data to select lines HEV101-16, HEV101-23, HEV110-27, and HEV110-28 for highest yields of recombinant protein.

Western blot of transgenic potato leaf extract showed that most of the recombinant HEV CP accumulated as 54 kDa, similar to the insect cell-derived antigen (Fig. 3). Some apparent proteolytic products of lower M_r were observed in both insect cell- and potato-derived material. HEV CP in plants transformed with either HEV101 or HEV110 showed similar patterns, with no qualitative or quantitative differences apparent. Failure to detect a larger protein for the single-truncation HEV101 (ΔN111) than that observed for the double truncation HEV110 (ΔN111/ΔC52) suggests that the ΔC52 truncation may occur in planta via an endogenous protease.

4. Discussion

HEV CP has been expressed in baculovirus-infected insect cell system and shown to assemble VLP [6,14]. The VLP have several advantages for the mucosal immunogen as follows: (1) rHEV VLP are composed of a single protein assembled into particles without nucleic acid. (2) rHEV VLP are easy to prepare and purify in a large quantities, approximately 1 mg per 2×10^7 insect cells. (3) rHEV VLP are antigenically similar to the native virion. (4) rHEV VLP are highly immunogenic in experimental animals when injected parenterally.

Our goal is to create transgenic plants that express HEV CP in edible tissues as VLP, in order to obtain an economical oral vaccine. It is likely that the success of oral delivery using VLP from insect cells is due to the particulate structure of the antigen, which contributed either to enhanced resistance to degradation in the gut or to enhanced uptake into the gut immune system.

In our studies with potato expressing HEV CP, we found very few VLPs, with the great majority of ELISA-positive antigen remaining near the top of a sucrose gradient (data not shown). Oral immunization of mice with potatoes expressing HEV CP failed to elicit detectable antibody responses in serum (data not shown). We extracted fecal pellets on day 18 after oral immunization on day 17 to evaluate the content of ELISA-reactive HEV capsid protein. Substantial antigen (3–4% of the dose) was present in pellets of mice that were fed HEV CP potato or gavaged with insect cell-derived VLP. Thus, the potato cells probably provided some protection to the soluble HEV CP present in potato tubers, and perhaps even limited uptake of antigen that may have been present as VLP. Since orally delivered insect cell-derived VLPs stimulated antibody responses and protected monkeys against HEV challenge [8], it is likely that poor VLP assembly in potato

was a major factor in the lack of oral immunogenicity of potato-derived HEV CP in mice. Future studies should focus on the optimization of VLP assembly in plant tissues, which may involve alternative plant host systems, and/or tissue and subcellular targeting of antigen.

Acknowledgements

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RESEARCH ARTICLE

DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration

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Delivery of foreign genes to the digestive tract mucosa by oral administration of nonreplicating gene transfer vectors would be a very useful method for vaccination and gene therapy. However, there have been few reports on suitable vectors. In the present study, we found that plasmid DNA can be packaged in vitro into a virus-like particle (VLP) composed of open reading frame 2 of hepatitis E virus, which is an orally transmissible virus, and that these VLPs can deliver this foreign DNA to the intestinal mucosa in vivo. The delivery of plasmid DNA to the mucosa of the small intestine was confirmed by the results of immunohistochemical analyses using an expression plasmid encoding human immunodeficiency virus env (HIV env) gp120. After oral administration of VLPs loaded with HIV env cDNA, significant levels of specific IgG and IgA to HIV env in fecal extracts and sera were found. Moreover, mice used in this study exhibited cytotoxic T-lymphocyte responses specific to HIV env in the spleen, Payer's patches and mesenteric lymph nodes. These findings suggest that VLPs derived from orally transmissible viruses can be used as vectors for delivery of genes to mucosal tissue by oral administration for the purpose of DNA vaccination and gene therapy.

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Keywords: *VLP; oral DNA vaccine; CTL; HIV; mucosal immunity*

Introduction

The successful outcome of novel gene therapies and DNA vaccinations largely depends on the development of effective delivery systems.[1] In human applications, both the efficacy and safety of any delivery system used for gene transfer are major concerns. It has been shown that tissue-specific gene transfer by a viral vector could be achieved naturally and effectively through cell specificity of the virus receptors.[2] However, there is a risk of vector toxicity through viral infection of the host cells. Also, the limited sizes of transgenes often present a serious obstacle. Nonviral vectors, such as liposomes, are safer but do not have a cell-specific targeting component and have limited transduction both *in vitro* and *in vivo*. This limitation has been partly overcome by the development of molecular conjugates consisting of cell-specific ligands that confer cell specificity to nonviral vectors.[3,4]

The development of a system for delivering genes to or conferring immunity to mucosal tissue by oral administration would provide a convenient means for effective treatment or prevention of various human diseases, including cancers, infectious diseases and immunological disorders.[5] Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al*[6]). However, there are several difficulties in oral immunization with nonreplicating molecules, such as low pH in the stomach, the presence of proteolytic enzymes in the digestive tract and the presence of physical as well as biochemical barriers associated with the mucosal surface itself.[6]

Among the various nonreplicating molecules, a virus-like particle (VLP), an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for vaccine development.[7] It is expected that the VLP structure will provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[8] However, VLPs can induce immune responses to themselves, and this is a problem for using VLPs as a vaccine vector to carry foreign DNA. A system using polyoma virus VP1 VLPs as a carrier of DNA by intranasal administration has been reported.[9] These VLPs work as an adjuvant, since DNA vaccine can induce immune responses by intranasal administration without VLPs. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes

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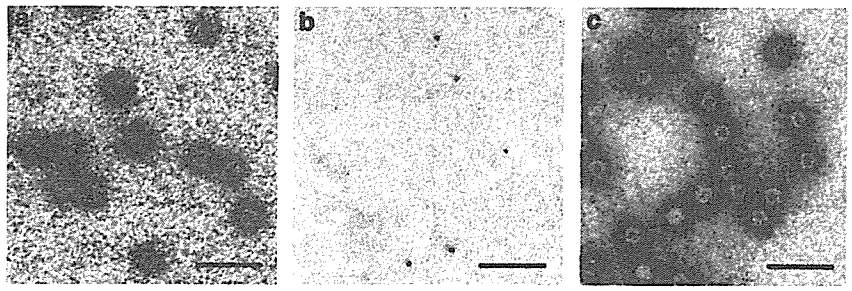

Figure 1 *Electron micrographs of HEV-VLPs: (a) purified HEV-VLPs before treatment; (b) disassembled HEV-VLPs after treatment of VLPs with EGTA and DTT; and (c) refolded HEV-VLPs in the presence of $CaCl_2$, DMSO and DNA. Bars represent 50 nm.*

human acute hepatitis by fecal–oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has recently been reported that overexpression of a part of open reading frame 2 (ORF2) in a baculovirus expression system results in the assembly of this protein into a VLP.[10] We have also reported that VLPs carrying foreign epitopes elicit strong mucosal and systemic immune responses to both the VLPs and exogenous epitopes without the requirement of any kind of adjuvant when orally administered to mice.[11]

Since infection with human immunodeficiency virus (HIV) most likely occurs through exposure of mucosal tissue to the virus, HIV-specific immune responses at mucosal sites are critical for the initial control of infection. Therefore, a nonreplicating vaccine vector that elicits mucosal immunity by oral administration would be a powerful HIV vaccine. In the present study, we found that unrelated plasmid constructs can be encapsulated into HEV-VLPs and delivered to the intestinal mucosa by oral administration. HIV DNA vaccine-loaded HEV-VLPs can elicit mucosal and systemic cellular as well as humoral immune responses by oral administration.

Results

In vitro refolding of VLPs

The HEV-VLPs produced by a recombinant baculovirus system were disassembled by the removal of calcium ions (Figure 1b). When calcium ions were supplemented to the disrupted VLPs in the presence of plasmid DNA, the DNA was encapsulated into the refolded VLPs (Figure 1c). No significant morphological difference due to the VLP disassembling–refolding process was observed under an electron microscope.

Density shifts of VLPs and amount of plasmid DNA after DNA encapsulation

Plasmid DNA encapsulation in the refolded VLPs was confirmed by CsCl equilibrium gradient centrifugation. VLP density is greater when loaded with a DNA plasmid. A heavier density gradient peak was present only when DNA was incorporated into the VLPs (Figure 2d). A single lighter density peak was produced for VLPs alone (Figure 2a), refolded VLPs (Figure 2b) and intact VLPs in the presence of plasmid DNA (Figure 2c). Despite the various sizes of plasmid DNA used for

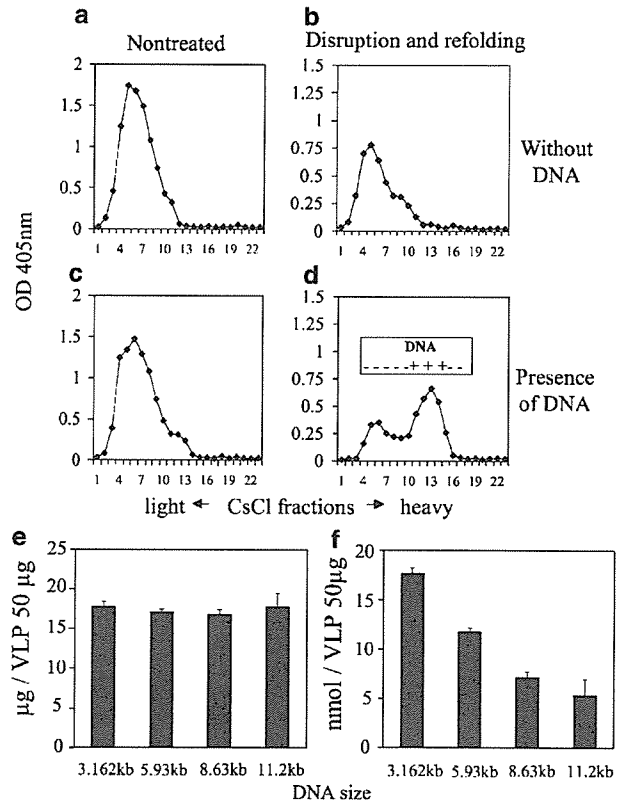

Figure 2 *CsCl gradient profiles of intact and refolded VLPs. No DNA added: (a) intact; (b) refolded. DNA added: (c) intact; (d) refolded. The amount of DNA encapsulated in VLPs is expressed as μg (e) and molality (f) per 50 μg VLP protein.*

encapsulation, the amounts of plasmid in VLPs were almost the same (17–19 μg per 50 μg of HEV-VLPs) (Figure 2e and f). A solution with a high concentration of plasmid DNA showed high viscosity, and VLPs including DNA were not obtained for general use in experiments. Based on these results, we used this amount (1 mg/ml) as the optimal concentration (data not shown).

Gene transfer by HEV-VLPs

Initially, four cell lines derived from mice, rabbits, monkeys and humans were studied for their ability to