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 coexpression 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 after the priming of the immune response appear to be an optimal window for augmentation of the immune response. Because 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.

GM-CSF has been reported to enhance antibody response (10) and plasmids GM-CSF has been used previously for this purpose. Coinoculation 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 coadministration of GM-CSF and human immunodeficiency virus type 1 (HIV-1) expression plasmids enhanced both Th1- and Th2-type responses (24). For a hepatitis B vaccine also both responses were affected by coexpression 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 coexpression 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 IgG_1 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. Because 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 fivefold higher than the amounts 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). Furthermore, in rhesus macaques immunized with HIV/HBsAg fusion proteins, injection of a HBsAg expressing plasmid 3 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) that 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.

ACKNOWLEDGMENTS

The authors are grateful to Dr. M.H. Tao (Taiwan) and Dr. O. Burrone (Italy) for the gift of IL-2 and GM-CSF expression plasmids, respectively.

REFERENCES

1. Arora, N.K., S.K. Nanda, S. Gulati, I.H. Ansari, M.K. Chawla, S.D. Gupta, and S.K. Panda. 1996. Acute viral hepatitis types E, A and B singly and in combination in acute liver failure in children in North India. J. Med. Virol. 48:215–221.
2. Aye, T.T., T. Uchida, X.-Z. Ma, F. Iida, T. Shikata, H. Zhuang, and K.M. Win. 1993. Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. Nucleic Acids Res. 20:3512.
3. Barouch, D.H., S. Santra, T.D. Steenbeke, X.X. Zheng, H.C. Perry, M.-E. Davis, D.C. Freed, A. Craiu, T.B. Strom, J.B. Shiver, and N.L. Letvin. 1998. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. J. Immunol. 161:1875–1882.
4. Bi, S.-L., M.A. Purdy, K.A. McCaustland, H.S. Margolis, and D.W. Bradley. 1993. The sequence of hepatitis E virus isolated directly from a single source during an outbreak in China. Virus Res. 28:233–247.
5. Borgne, S.L., M. Mancini, R.L. Grand, M. Schleaf, D. Dormant, P. Tiollais, Y. Riviere, and M.-L. Michel. 1998. In vivo induction of specific cytotoxic T lymphocytes in mice and Rhesus macaques immunized with DNA vector encoding an HIV epitope fused with hepatitis B surface antigen. Virology. 240:304–315.
6. Chow, Y-H., B.-L. Chiang, Y.-L. Lee, W.-K. Chi, W-C. Lin, Y.-T. Chen, and M.-T. Tao. 1998. Development of Th1 and Th2 populations and the nature of immune responses to hepatitis B virus DNA vaccines can be modulated by co-delivery of various cytokine genes. J. Immunol. 160:1320–1329.
7. Chow, Y.-H., W.-L. Huang, W.-K. Chi, Y.-D. Chu, and M.-H Tao. 1997. Improvement of hepatitis B virus DNA vaccines by plasmids co-expressing hepatitis B surface antigen and interleukin-2. J. Virol. 71:169–178.
8. Davis, H.L., Mancini, M.-L. Michel, and R.G. Whalen. 1996. DNA-mediated immunization to hepatitis B surface antigen: Longevity of primary response and effect of boost. Vaccine. 14:910–915.
9. Davis, H.L., M.J. McCluskie, J.L. Gerin, and R.H. Purcell. 1996. DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proc. Natl. Acad. Sci. USA. 93:7213–7218.
10. Disis, M.L., H. Bernard, F.M. Shiota, S.L. Hand, J.R. Gralow, E.S. Huseby, S. Gillis, and M.A. Cheever. 1996. Granulocyte-macrophage colony stimulating factor: An effective adjuvant for protein and peptide based vaccine. Blood. 88:202–210.
11. Donnelly, J.J., J.B. Ulmer, J.W. Shiver, and M.A. Liu. 1997. DNA vaccines. Annu. Rev. Immunol. 15:617–648.
12. Geissler, M., A. Gesien, K. Tokushige, and J.R. Wands. 1997. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. J. Immunol. 158:1231–1237.
13. He, J., S.L. Hoffman, and C.G. Haves. 1997. DNA inoculation with a plasmid vector carrying the hepatitis E virus structural protein gene induces immune response in mice. Vaccine. 15:357–362.
14. Huang, C.-C., D. Nguyen, J. Fernandez, K.Y. Yun, K.E. Fry, D.W. Bradley, A.W. Tam, and G.R. Reyes. 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology. 191:550–558.
15. Jameel, S., M. Zafrullah, M.H. Ozdener, and S.K. Panda. 1996. Expression in animal cells and characterization of the hepatitis E virus structural proteins. J. Virol. 70:207–216.
16. Kaur, M., K.C. Hvams, M.A. Purdy, K. Krawczynski, W.M. Ching, K.E. Fry, G.R. Reves. D.W. Bradley, and M. Carl. 1992. Human linear B-cell epitopes encoded by the hepatitis E virus includes determinants in the RNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. USA. 89:3855–3858.
17. Khudyakov, Y.E., M.O. Favorov, D.L. Jue, T.K. Hine, and H.A. Fields. 1994. Immunodominant antigenic regions in a structural protein of the Hepatitis E virus. Virology. 198:390–393.
18. Kuroo, M.S., M.R. Teli, S. Skidmore, M.A. Sofi, and M.I. Khuroo. 1981. Incidence and severity of viral hepatitis in pregnancy. Am. J. Med. 70:252–255.
19. Krawczynski, K. 1993. Hepatitis E. Hepatology 17:932–941.
20. Li, T.-C., Y. Yamakawa, K. Suzuki, M. Tatsumi, M.A.A. Razak, M.T. Uchida, N. Takeda, and T. Miyamura. 1997. Expression and self assembly of empty virus-like particles of hepatitis E virus. J. Virol. 71:7207–7213.
21. McDonnell, W.M. and F.K. Askari. 1996. DNA vaccines. N. Engl. J. Med. 334:42–45.

22. Meng, J., J. Pillot, X. Dai, H.A. Fields, and Y.E. Khudyakov. 1998. Neutralization of different geographic strains of the hepatitis E virus with anti-hepatitis E virus-positive serum samples obtained from different sources. Virology. 249:316–324.

23. Nunberg, J.H., M.V. Doyle, S.M. York, and C.J. York. 1989. Interleukin-2 acts as an adjuvant to increase the potency of inactivated rabies virus vaccine. Proc. Natl. Acad. Sci. USA. 86:4240–4243.

24. Okada, E., S. Sasaki, N. Ishii, I. Aoki, T. Yasuda, K. Nishioka, J. Fukushima, J.-I. Miyazaki, B. Wahren, and K. Okuda. 1997. Intranasal immunization of a DNA vaccine with IL-12 and granulocyte-macrophage colony stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. J. Immunol. 159:3638–3647.

25. Oppenheim, J.J., C.O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. 9:617–648.

26. Panda, S.K., and S. Jameel. 1997. Hepatitis E virus: From epidemiology to molecular biology. Viral. Hep. Rev. 3:227–251.

27. Panda, S.K., S.K. Nanda, H. Durgapal, H. Ozdner, M. Zafrullah, I.H. Ansari, R.S. Mehra, and S. Jameel. 1997. Diagnostic and protective immune status of hepatitis E virus structural antigens, pp 321–326, in: Viral hepatitis and liver disease ed. M. Rizzetto, R.H. Purcell, J.L. Gerin, and G. Verme, eds. Edizioni Minerva Medica. Turin.

28. Tam, A.W., M.M. Smith, M.E. Guerra, C.-C. Huang, D.W. Bradley, K.E. Frey, and Reyes, G.R. 1991. Hepatitis E virus (HEV): Molecular cloning and sequencing of the full length viral genomes. Virology. 185:120–131.

29. Tsarev, S.A., S.U. Emerson, G.R. Reves, T.S. Tsareva, L.J. Legters, A. Malik, M. Iqbal, and R.H. Purcell. 1992. Characterization of a prototype strain of hepatitis E virus. Proc. Natl. Acad. Sci. USA. 89:559–563.

30. Tsarev, S.A., T.S. Tsareva, S.U. Emerson, S. Govindrajan, M. Shapiro, J.L. Gerin, and R.H. Purcell. 1994. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proc. Natl. Acad. Sci. USA. 91:10198–10202.

31. Tuteja, R. 1999. DNA vaccines: A ray of hope. Crit. Rev. Biochem. Mol. Biol. 34:1–24.

32. Ulmer, J.B., J.J. Donnelly, and M.A. Liu. 1996. Toward the development of DNA vaccine. Curr. Opin. Biotechnol. 7:653–658.

33. Wang, Y., R. Ling, J.C. Erker, H. Zhang, H. Li, S. Desai, I.K. Mushahwar, T.J. Harrison. 1999. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J. Gen. Virol. 80:169–177.

34. Weinberg, A., and T.C. Merigen. 1988. Recombinant interleukin-2 as an adjuvant for vaccine-induced protection: Immunization of guinea pigs with herpes simplex virus subunit vaccines. J. Immunol. 140:294–299.

35. Xiang, Z., and H.C.J. Ertl. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmid expressing cytokines. Immunity. 2:129–135.

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Received December 6, 1999; revised February 3, 2000; accepted February 3, 2000.

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Vaccine 00 (2001) 000–000

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Oral administration of hepatitis E virus-like particles induces a systemic and mucosal immune response in mice

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Received 21 December 2000; received in revised form 29 January 2001; accepted 29 January 2001

Abstract

We evaluated the potential of recombinant hepatitis E virus (rHEV) virus-like particles (VLPs) as an oral immunogen by analyzing the response of serum IgM, IgG, and IgA and fecal IgA in mice after oral administration. 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 VLPs. Mice were orally inoculated four times with purified rHEV VLPs in concentrations ranging from 10 to 100 μg without adjuvant. Serum IgM response was obtained with as little as 10 μg of the VLPs, and the level reached its maximum in all mice groups within 2 weeks after the first administration. Serum IgG was detected by 4 weeks post-immunization (p.i.) in the majority of mice given doses of 50 and 100 μg and continuously increased at least until the 10 week mark. Serum IgA was also detected by 4 weeks p.i. in the majority of mice given doses of 50 and 100 μg, and the level reached the maximum at 8 weeks p.i.. Furthermore, the maximum level of intestinal IgA responses was detected in the groups of mice receiving 50 and 100 μg rHEV VLPs at 8 weeks p.i. All these antibody responses were obtained without a mucosal adjuvant. We therefore concluded that oral immunization of rHEV VLPs is capable of inducing systemic as well as intestinal antibody responses. Furthermore, serum IgG and fecal IgA thus induced were reactive to the native HEV antigen, as determined by Western blot assays and antigen-capture ELISA.

Keywords: Hepatitis E virus; Oral administration; Virus-like particles

1. Introduction

Infection with hepatitis E virus (HEV) causes acute hepatitis. The virus is transmitted via an oral–fecal route, often by contaminated drinking water [1,2]. Hepatitis E occurs predominantly in developing countries, usually affecting young adults, and the mortality rate is very high, up to 15–20%, in infected pregnant women [3–5]. In developed countries, most cases have been imported [6,7].

The symptoms of HEV infection are self-limited, generally lasting 1–2 months, requiring hospitalization and proper therapy [8]. Infection by HEV is limited to the hepatocyte, and viremia is always observed prior to the manifestation of the disease [9]. Serum antibody is thought to be sufficient to prevent the disease [10]; however, because the primary site of HEV infection is the intestine, the induction of local immunity may be important for protection against infection and disease. Of all the immunoglobulins in the intestinal tract, immunoglobulin A (IgA) is the predominant species of antibody, and it functions through building mucosal immunity [11]. Therefore, an effective HEV vaccine should have the ability to induce a specific intestinal IgA response.

No practical cell-culture system to allow the growth of HEV has been developed [5]; however, molecular cloning of the HEV genome revealed that the genomic RNA appears to encode a single capsid protein [12]. When the capsid protein, with 111 amino acids truncated at the N-terminal, was expressed in the baculovirus expression system, it was spontaneously assembled into virus-like particles (VLPs) [13]. Electron cryomicroscopy study shows that these VLPs are formed with 60 copies of a 50 kDa protein arranged in T = 1 symmetry [14]. The VLPs have several advan-

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0264-410X/01/$ - see front matter
PII: S0264-410X(01)00059-7

tages as the mucosal immunogen, as follows: (1) the recombinant HEV (rHEV) VLPs are composed of a single protein assembled into particles without nucleic acid, which makes them unable to replicate; (2) rHEV VLPs are easy to prepare and purify in large quantities, with a yield of approximately 1 mg per 10^7 insect cells; (3) rHEV VLPs are antigenically similar to the native virion; (4) rHEV VLPs are highly immunogenic in experimental animals when injected parenterally; (5) rHEV VLPs are stable at a low pH (such as the pH of the stomach); (6) natural HEV infection occurs via the oral–fecal route, so oral delivery of rHEV VLPs could induce the same immune responses as occur in natural infection [13,14].

The oral immunization of recombinant VLPs to prevent disease has been described previously. For example, immunologically naïve dogs immunized with recombinant canine oral papillomavirus (COPV) VLPs are capable of inducing serum antibodies that could protect dogs against the challenge of COPV [15]. Similar experiments have been done with cottontail rabbit papillomavirus [16], bovine papillomavirus [17], human papillomavirus [18], and Norwalk virus [19,20].

In order to assess the potential of rHEV VLPs as an oral immunogen, we analyzed the response of serum IgM, IgG, and IgA and fecal IgA in mice after oral administration of rHEV VLPs. The rHEV VLPs were shown to be immunogenic, generating high titers of HEV-specific antibody in serum that was capable of binding to the native HEV antigen. Oral, but not parenteral, immunization was shown to be effective in producing HEV-specific antibodies in mucosa.

2. Materials and methods

2.1. Preparation and purification of rHEV VLPs

The molecular cloning and construction of a recombinant baculovirus Ac5480/7126 harboring the capsid protein gene lacking 111 amino acids at the N-terminal were described previously [13]. The rHEV VLPs were prepared using Tn5 cells (High Five™, Invitrogen, San Diego, CA) 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 VLPs were concentrated by centrifugation for 2 h at 100 000 × *g* in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA) and purified by isopycnic binding in CsCl gradient. A visible band containing rHEV VLPs was collected, and the rHEV VLPs were diluted five times with phosphate-buffered saline solution (PBS)(−). The rHEV VLPs were pelleted at 100 000 × *g* and then resuspended in PBS(−). Purity of the rHEV VLPs was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electron microscopy after negative staining, as described previously [13].

2.2. Oral delivery of the rHEV VLPs

Three dose levels of rHEV VLPs [10, 50, and 100 μg in 500 μl PBS(−)] were administered intragastrically to three groups of female BALB/c mice (CLEA Japan, Inc., Tokyo) aged 8 weeks. A control group of mice received PBS(−). Pre-inoculation serum and fecal samples were collected before the first administration. Each mouse was given four doses of rHEV VLPs on days 0, 12, 24, and 48 by oral gavage using a plastic intubation needle (CLEA Japan, Inc.). In some experiments, a fifth immunization was administered on day 80.

2.3. Sample collection

Blood samples were obtained by tail bleeding at 1 week intervals following the primary inoculation. Fecal samples were collected from individual mice and extracted by making a 1:10 suspension (wt/vol) with PBS(−). The suspension was vortexed and centrifuged for 10 min at 12 000 × *g*. The supernatant was collected and stored at −20°C until tested.

2.4. Antibody enzyme-linked immunosorbent assay (ELISA)

HEV-specific serum IgM, IgG, and IgA and fecal IgA antibody titers were determined by ELISA using rHEV VLPs as the antigen. Flat-bottomed 96-well polystyrene microplates (Immulon 2, Dynex Technologies, Inc. Chantilly, VA) were coated with the purified VLPs, and serum samples (100 μl/well) were added in duplicate at twofold serial dilutions. For the detection of fecal IgA, the supernatant of the extract was diluted in the same manner as those used for the serum specimens. The plates were then incubated for 1 h at 37°C, washed six times, and administered with 100 μl of horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA) (1:2000 dilution) or IgM (Zymed Laboratories, South San Francisco, CA) (1:2000 dilution) in PBS(−) containing 0.05% of Tween-20 (PBS-T) and 1% skim milk. We used HRP-conjugated rabbit anti-mouse IgA (Zymed) (1:2000 dilution) for the IgA. The plates were incubated for 1 h at 37°C and washed six times with PBS-T, and then 100 μl of the substrate orthophenylenediamine (Sigma Chemical, Co., St. Louis, MO) were added to each well. The plates were incubated in a darkroom for 30 min at room temperature, and then 50 μl of 4 N H_2SO_4 were added to each well. After the plates had been stood at room temperature for 10 min, the absorbance at 492 nm was measured.

We assayed several twofold dilutions and used the reciprocal of the highest dilution that had an absorbance greater than or equal to 0.15 above the background as the antibody titer. Geometric mean titers (GMTs) and standard errors were calculated on the log-transformed antibody titers.

2.5. Western blot assay

Approximately 1 μg of rHEV VLPs protein was separated by SDS–PAGE, and 54k protein was detected by Western blot analysis with either a pooled mouse sera (1:100 dilution) or pooled 10% suspension of the fecal extract (1:5 dilution) collected at 54 days after immunization. Detection of serum IgG antibody was achieved by using alkaline phosphatase coupled with goat anti-mouse IgG (1:2000 dilution) (EY Laboratories, Inc., San Mateo, CA). Detection of fecal IgA antibody was done with alkaline phosphatase coupled with goat anti-mouse IgA (1:2000 dilution) (Southern Biotechnology Associates, Inc., Birmingham, AL). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA).

2.6. Antigen-capture ELISA

Microplates were coated with either 100 μl of the rabbit hyperimmune serum (1:5000 dilution) to rHEV VLPs or an equal amount of the preimmune serum [13]. Twofold dilution (100 μl) of 10% suspension of the stool specimen from an experimentally infected monkey was added to duplicate wells and incubated for 1 h at 37°C. The stool specimen was infectious and contained the native HEV antigens (data not shown). Either 100 μl of pooled sera (1:100 dilution) or 100 μl of pooled 10% stool suspension (1:2 dilution) collected at 54 days post-immunization (p.i.) were added and incubated for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG and IgA described in the antibody ELISA were used to detect bound subclass antibodies to the native virion.

3. Results

3.1. Characterization of rHEV VLPs

The rHEV VLPs were generated by a recombinant baculovirus harboring the gene for the capsid protein with 111 amino acids truncated at the N-terminal using insect Tn5 cells [13]. The rHEV VLPs were purified by centrifugation and characterized by SDS–PAGE and Western blot assay, where a major protein band with a molecular weight of 54k was observed (Fig. 1A). This 54k protein was characterized as a 50k protein in previous studies due to the use of different electrophoresis conditions [13,21]. Electron microscopy showed a large number of VLPs (Fig. 1B) that were morphologically identical to those used for the electron cryomicrography that were described previously [14].

3.2. Kinetics of serum IgM, IgG, and IgA responses

To evaluate the potential of the rHEV VLPs as an oral immunogen, we administered three doses of the rHEV VLPs (10, 50, and 100 μg) to inbred BALB/c mice by oral gavage on days 0, 12, 24, and 48. All of the administration was carried out without adjuvant, and the serum antibody responses were monitored by ELISA (Fig. 2). The geometric mean titer was calculated for each group of mice as described in Materials and methods, with all of the nonresponders included in

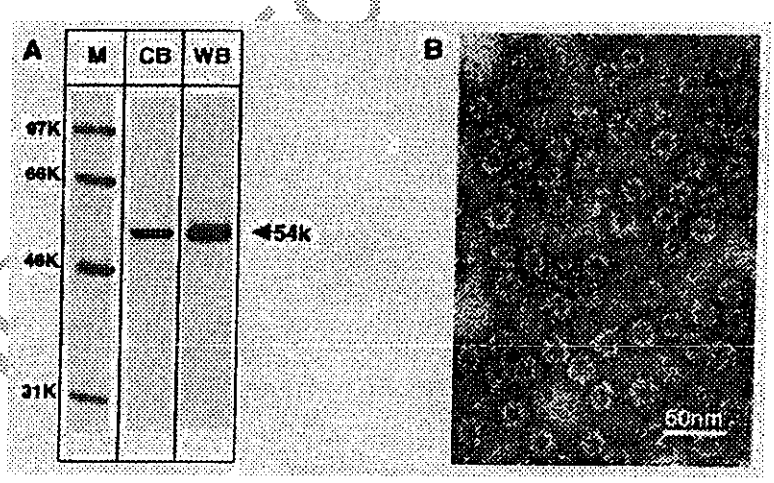

Fig. 1. Characterization of the rHEV VLPs. (A) The recombinant capsid protein spontaneously assembled into VLPs was separated by 10% SDS–PAGE followed by staining with Coomassie Brilliant Blue (CB) and Western blot assay using a serum from a hepatitis E patient (WB). (B) Electron microscopy of rHEV VLPs purified from Tn5 cells by CsCl equilibrium centrifugation. The VLPs were stained with 2% uranyl acetate.

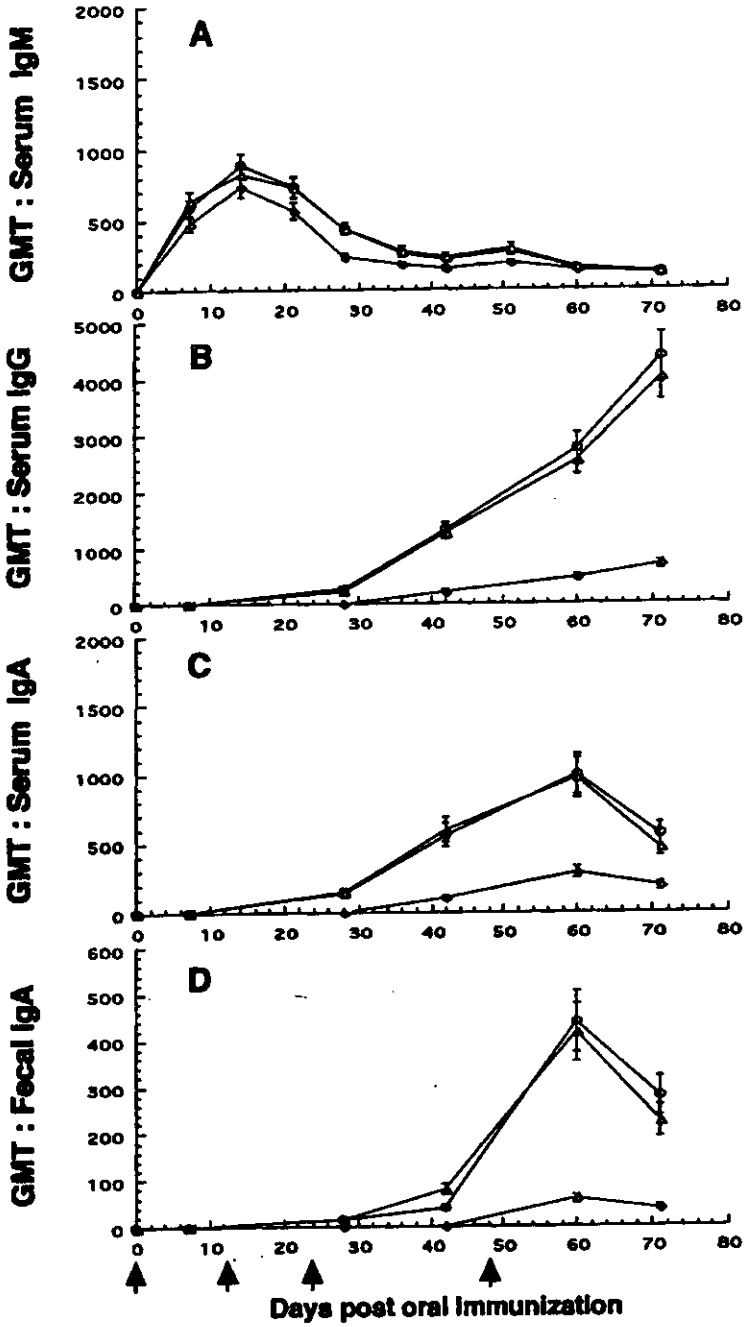

Fig. 2. Kinetics of serum IgM (A), IgG (B), and IgA (C) and fecal IgA (D) antibody responses of BALB/c mice after oral immunization with rHEV VLPs. The *y*-axis shows the GMT of the antibody for doses of 10 μg (8 mice; —◇—), 50 μg (9 mice; —△—), and 100 μg (7 mice; —○—), and the *x*-axis shows the number of days post immunization. The VLPs were given orally on days 0, 12, 24, and 48, as indicated by arrows. The error bars show the standard errors of the mean.

the computation. All of the preimmune samples taken prior to the first immunization were negative for IgM, IgG, and IgA, showing geometric mean titers (GMTs) less than 100. Control groups given PBS(−) were also negative throughout the experiments (GMTs < 100).

Serum IgM response was obtained with as little as 10 μg of the particles and reached a maximum in all mice groups within 2 weeks after the first administration, with a GMT of 738 (Fig. 2A). Serum IgM antibody titers induced by 50 and 100 μg of the VLPs were very

similar, only slightly exceeding the titers induced by 10 μg of the VLPs. The IgM antibody titers decreased gradually and reached the basal level in 9 weeks. Interestingly, the titers were slightly increased after the fourth immunization.

A serum IgG response was detected at 4 weeks, continuously increasing at least until 10 weeks in the groups of mice given doses of 50 and 100 μg, whereas it was detected at 6 weeks after the first administration in a group of mice given a dose of 10 μg of rHEV VLPs. The serum IgG titers induced by 50 and 100 μg of VLPs at 8 weeks (on day 60) were variable, ranging from 320 to 10 240, with GMTs of 2562 and 2758, respectively (Fig. 2B).

Serum IgA responses in the groups of mice receiving 50 and 100 μg VLPs reached a maximum at 8 weeks (on day 60), ranging from 100 to 6400 with GMTs of 970 and 998, respectively. The antibody responses to 10 μg of VLPs were significantly lower throughout the experiments ($P < 0.01$) (Fig. 2C). These data demonstrated that the rHEV VLPs are immunogenic when delivered orally to mice and that a mucosal adjuvant is not required for the induction of serum IgM, IgG, or IgA.

3.3. Oral immunization of rHEV VLPs induces an intestinal IgA response in mice

To evaluate the potential of the rHEV VLPs to induce a mucosal antibody, fecal specimens were tested for IgA by ELISA (Fig. 2D). A control group of mice given PBS(−) were negative for IgA (GMTs < 10). Intestinal IgA responses were similar to those of serum IgA. A dose of 10 μg of VLPs elicited significantly lower IgA titers when compared with those induced by 50 and 100 μg doses. Intestinal IgA responses in the groups of mice receiving 50 and 100 μg VLPs reached a maximum at 8 weeks (on day 60), ranging from 40 to 1280 with GMTs of 416 and 438, respectively. These data indicated that the rHEV VLPs are immunogenic when given orally to mice and that a mucosal adjuvant is not required for the induction of an intestinal IgA antibodies. Relatively large doses of VLPs were required to induce IgA antibody responses when given orally to mice.

3.4. Comparison of serum IgG, serum IgA, and fecal IgA responses in mice given rHEV VLPs either orally or parenterally

We investigated the effect of the delivery route of the rHEV VLPs on immune responses in three groups of BALB/c mice. Doses of 10 and 50 μg of the rHEV VLPs were delivered by intraperitoneal (i.p.) injections on days 0, 22, and 44. For comparison, 50 μg of the VLPs were delivered by four oral doses on days 0, 11, 22, and 44, with a subsequent dose delivery on day 80 (Fig. 3). Serum IgG antibodies were efficiently induced within 1 week post-i.p. immunization. Serum IgG antibodies elicited by oral immunization slowly increased, as shown in Fig. 2B, and reached a relatively high titer 45 days after the first immunization (Fig. 3A). Serum IgA antibodies were elicited only when rHEV VLPs were given by oral immunization (Fig. 3B). There was a significant difference in the IgA responses based on the

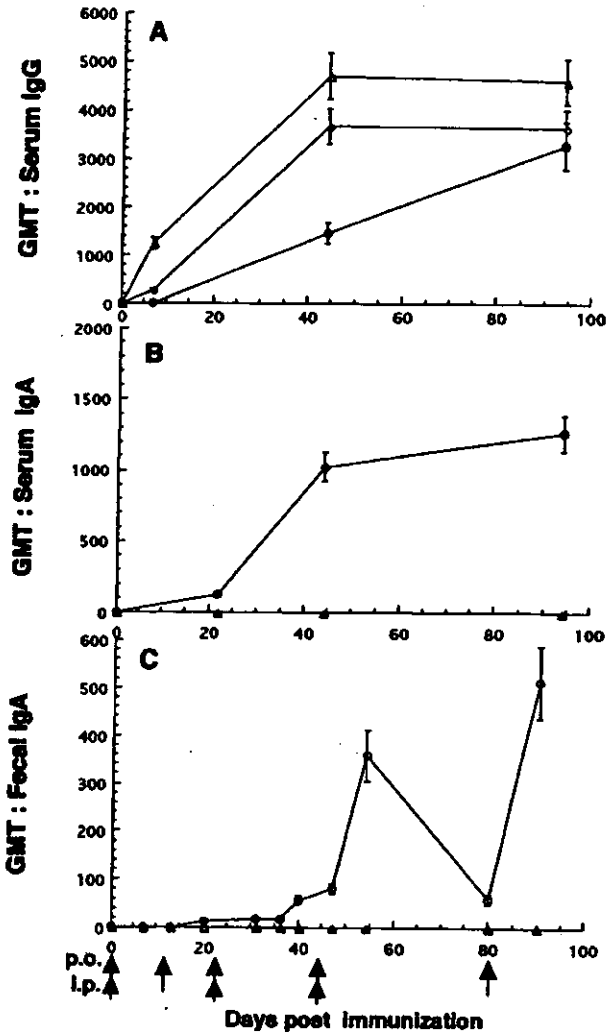

Fig. 3. Comparison of serum IgG (A), serum IgA (B), and fecal IgA (C) antibody responses of BALB/c mice after oral (p.o.) and intraperitoneal (i.p.) immunization of rHEV VLPs. The y-axis shows the GMT of the antibody for 10 μg of i.p. inoculation with 10 mice (---◇---), 50 μg of i.p. inoculation with 10 mice (---△—), and 50 μg of p.o. inoculation with 12 mice (—○—), and the x-axis shows the number of days post immunization. The VLPs were given either orally or by intraperitoneal injection on the days indicated by arrows. Serum IgG, serum IgA, and fecal IgA antibody responses were measured by ELISA. The error bars show the standard errors of the mean.

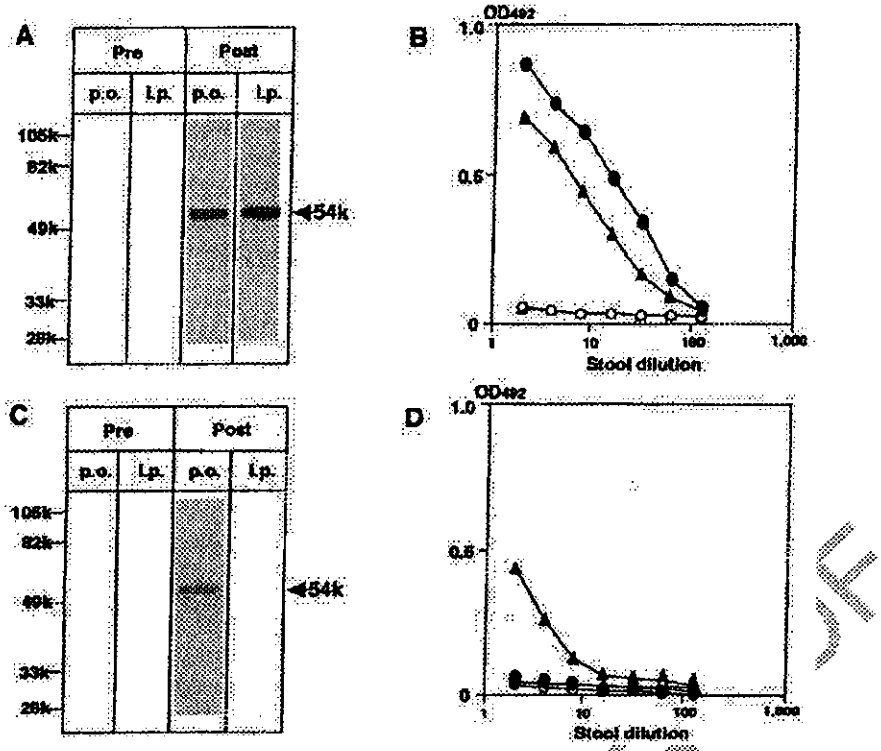

Fig. 4. Reactivity and specificity of serum IgG and fecal IgA elicited against orally delivered rHEV VLPs as determined by Western blot assay and antigen capture ELISA. (A) A 54k protein was detected by Western blot analysis with pooled sera from either orally (p.o.) or intraperitoneally (i.p.) immunized mice (Post). Pooled sera from preimmunized mice were included as a negative control (Pre). (B) Serum IgG bound to the native HEV antigens was measured by an antigen-capture ELISA with a fecal specimen from experimentally infected (closed symbols) or preinfected (open symbols) monkeys. (C) The same experiments as (A) were carried out with pooled 10% stool suspension. (D) Fecal IgA bound to the native HEV antigens were measured in the same manner as (B) with pooled 10% stool suspension. Antigen from a preinfected monkey and detector antibody from a mouse with i.p. immunization; ---○---, Antigen from a preinfected monkey and detector antibody from a mouse with p.o. immunization; ---△---, Antigen from an infected monkey and detector antibody from a mouse with i.p. immunization; —●—, Antigen from an infected monkey and detector antibody from a mouse with p.o. immunization; —▲—.

route of the antigen delivery. No serum IgA antibody was elicited in mice when antigens were given by i.p. injection, even when a dose of 50 μg was used. Similarly, no intestinal IgA antibody was elicited in mice when antigens were given by i.p. injection (Fig. 3C). It should also be noted that intestinal IgA antibody titers decreased very rapidly, reaching the basal level at day 80, but this low level of IgA antibody increased very rapidly and reached high titers with GMTs of 512 in 10 days after the fifth immunization at day 80.

3.5. Characterization of serum and intestinal antibodies induced by orally delivered rHEV VLPs

We tested the reactivity of the serum IgG antibody elicited by oral immunization by Western blot analysis (Fig. 4A). No reactivity was found in pooled preimmune sera from either p.o. or i.p. immunization. Pooled mouse sera collected at 8 weeks after the i.p administration gave a strong band with a molecular weight of 54 k, corresponding to the N-terminal-deleted capsid protein. A predominant signal was also found in the same position when pooled sera from 8 weeks after p.o. immunization were tested. These data indicated that the serum IgG antibodies elicited against orally delivered rHEV VLPs were as reactive to rHEV VLPs as those elicited against i.p.-immunized rHEV VLPs.

To further characterize the mouse antibody induced by the orally administered rHEV VLPs, we tested the reactivity to the native HEV antigen with an antigen-capture ELISA (Fig. 4B). A microplate was coated with a rabbit hyperimmune serum (ELISA titer 1:1 000 000) raised against rHEV VLPs, as previously described [13], and twofold dilutions of a fecal specimen from a cynomorgus monkey infected with 10% suspension of a fecal sample from an acute hepatitis E patient were added. This monkey fecal specimen was HEV positive as indicated by reverse transcription-polymerase chain reaction (RT-PCR) and antigen ELISA. This specimen was also infectious when a 10 000-times-diluted sample was injected intravenously into other cynomorgus monkeys (data not shown), so we knew that it contained

infectious HEV particles. We evaluated the reactivity of the antibodies in the pooled mouse sera, which were bound to the native HEV antigen, by using these sera as the detector antibody. Pooled sera were negative in the ELISA when the fecal specimen from the preinfected monkey was used as the antigen source. In contrast, high OD values were obtained when the pooled sera from i.p. immunized mice were tested. High OD values were also obtained when the pooled sera from p.o. immunized mice were tested. These data indicated that mouse serum IgG elicited against the orally delivered rHEV VLPs was specific to HEV and capable of binding to the native HEV antigen.

Similar results were obtained by both Western blot assay and antigen-capture ELISA when IgA antibody in the pooled fecal suspensions from p.o.-immunized mice was monitored (Fig. 4C and D). In contrast, neither a 54k band nor an OD value was detected by Western blot assay or antigen capture ELISA when IgA antibody response was evaluated with the pooled fecal specimen from i.p.-immunized mice. These results demonstrated that an HEV-specific fecal IgA response was elicited only when HEV-VLPs were administered via the oral route.

4. Discussion

Nearly all viral infections gain entrance through mucosal surfaces. As the infection by HEV occurs via the oral–fecal route [1,2,5], it is important to develop vaccines that induce protective immune responses at the luminal surface of the intestinal tract as a first line of defense. Furthermore, since viremia of HEV preceding the development of the acute phase of illness is thought to occur during the incubation period [5,22], the vaccine should not only produce a local immune response at the surface of the intestinal tract but also elicit HEV-specific immunity in the systemic lymphoid tissues. Here, we studied the serum IgM, IgG, and IgA and intestinal IgA responses in mice given rHEV VLPs orally. Though the protective immunity was not assessed because mice are not susceptible to infection with HEV, and neutralization activity of the antibodies induced by the VLPs was not tested because no practical cell-culture system to allow the growth of HEV has been developed, the results of this study provided a model for evaluating the potential of rHEV VLPs as an oral subunit vaccine.

Cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have often been used as mucosal adjuvants to induce a strong mucosal immune response. In the oral immunization of mice using recombinant Norwalk virus (NV) VLPs, CT was shown to influence the magnitude of the IgG response at higher doses, but CT was not required for the induction of a systemic and mucosal antibody response [19]. A recent phase I study with rNV indicated that orally administered rNV VLPs are immunogenic in healthy adults when given without adjuvant [23]. Although LT is less toxic than CT, there is still a potential to induce diarrhea, limiting its benefits as a mucosal adjuvant [24–26]. Induction of systemic virus-neutralizing antibodies to human papillomavirus has been achieved when VLPs were orally administered to mice without adjuvants [18]. We report here that oral administration of rHEV VLPs was immunogenic and stimulated an immune response in mice in the absence of an adjuvant.

IgA is the predominant antibody at the mucosal surface. It is locally produced at a level that exceeds that of all of the other immunoglobulins and is important for mucosal immunity. Therefore, it is likely that to be effective, an oral HEV vaccine will have to induce a specific intestinal IgA response [27]. Stimulation of local precursor IgA plasma cells might be efficient because rHEV VLPs is particulate. The three-dimensional structure of an rHEV VLPs particle revealed by cryoelectron microscopy showed that the capsid is dominated by a dimer that defines the 30 morphological units [14]. When rHEV VLPs were delivered by the intestinal route, the particles might have been highly immunogenic, even in the absence of adjuvant, due to their particulate and repetitive antigenic structure. We hypothesized that rHEV VLPs might be targeted to gut-associated lymphoid tissue (GALT) via M cells of the Peyer's Patch (PP) and actively pinocytosed from the gut lumen, or mediated by a specific cellular receptor to cross the mucosal epithelia. They are then transported to underlying lymphoid cells, where they activate the mucosal immune responses [28]. M cell-mediated uptake into the PP has been demonstrated in a number of pathogens that invade via the surface of the intestinal tract [29–34].

Oral tolerance is of major concern for mucosal immunization, since the systemic immunological unresponsiveness that occurs after feeding soluble antigen or induced by feeding antigen prior to parenteral immunization must be overcome. Though multiple mechanisms of tolerance have been demonstrated, the generation of suppressor $CD8^+$ T cells that may secrete TGF-β, an inhibitory cytokine, is the most common mechanism [27]. However, recent accumulated results provided supportive evidence for the inhibitory role of TGF-β producing Th3 cells, and IL-10 and TGF-β producing Tr1 type cells instead of TGF-β secreting $CD8^+$T cells [35]. Our experiments demonstrated that oral immunization with the rHEV VLPs did not induce oral tolerance because the GMT titers of IgG and IgA continuously increased after each oral immunization (Fig. 2), and the number of the responder also increased (data not shown). Furthermore, a rapid and drastic increase of intestinal IgA was observed follow-

ing the fifth immunization (Fig. 3). This is because rHEV VLPs were administered to mice as particles in which the capsid protein might have been folded in a way that allowed it to maintain a structure prohibiting oral tolerance.

Oral immunization offers many advantages over parenteral immunization, including easy delivery, more acceptability to recipients, reduction in the purity, and reduction in the number of trained personnel needed to administer injections, all of which ultimately reduce production costs. The rHEV VLPs given orally provide a non-replicate and safe candidate vaccine for HEV.

Acknowledgements

This work was supported in part by grants-in-aid from Research on Pharmaceutical and Medical Safety, Health Sciences Research Grants, the Ministry of Health and Welfare, Japan.

References

[1] Khuroo MS. Study of an epidemic of non-A, non-B hepatitis: possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am J Med 1980;68:818–24.
[2] Wong DC, Purcell RH, Sreenivasan MA, Prasad SR, Pavri KM. Epidemic and endemic hepatitis in India: Evidence for a non-A, non-B hepatitis virus aetiology. Lancet 1980;2:876–9.
[3] Kane MA, Bradley DW, Shrestha SM, Maynard JE, Cook EH, Mishra RP, Joshi DD. Epidemic non-A, non-B hepatitis in Nepal. Recovery of a possible etiologic agent and transmission studies in marmosets. J Am Med Assoc 1984;252:3140–5.
[4] Khuroo MS, Teli MR, Skidmore S, Sofi MA, Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. Am J Med 1981;70:252–5.
[5] Purcell RH. Hepatitis E virus. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE, editors. Fields Virology, vol. 2, third ed. Philadelphia, PA: Lippincott-Raven Publishers, 1996:2831–43.
[6] De Cock KM, Bradley DW, Sandford NL, Govindarajan S, Maynard JE, Redeker AG. Epidemic non-A, non-B hepatitis in patients from Pakistan. Ann Intern Med 1987;106:227–30.
[7] Fortier D, Treadwell TL, Koff RS. Enterically transmitted non-A, non-B hepatitis: importation from Mexico to Massachusetts [letter]. New Engl J Med 1989;320:1281–2.
[8] Balayan MS, Andjaparidze AG, Savinskaya SS, Ketiladze ES, Braginsky DM, Savinov AP, Poleschuk VF. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 1983;20:23–31.
[9] Chauhan A, Jameel S, Dilawari JB, Chawla YK, Kaur U, Ganguly NK. Hepatitis E virus transmission to a volunteer. Lancet 1993;341:149–50.
[10] Khuroo MS, Kamili S, Dar MY, Moecklii R, Jameel S. Hepatitis E and long-term antibody status [letter]. Lancet 1993;341:1355.
[11] Mestecky J, McGhee JR. Immunoglobin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. In: Dixon FJ, editor. Adv. Immunol, vol. 40. San Diego, CA: Academic Press, 1987:153–245.
[12] Reyes GR, Huang CC, Tam AW, Purdy MA. Molecular organization and replication of hepatitis E virus (HEV). Arch Virol Suppl 1993;7:15–25.
[13] Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 1997;71:7207–13.
[14] Xing L, Kato K, Li T, Takeda N, Miyamura T, Hammar L, Cheng RH. Recombinant hepatitis E capsid protein self-assembles into a dual- domain T = 1 particle presenting native virus epitopes. Virology 1999;265:35–45.
[15] Suzich JA, Ghim SJ, Palmer-Hill FJ, White WI, Tamura JK, Bell JA, Newsome JA, Jenson AB, Schlegel R. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA 1995;92:11553–7.
[16] Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trin-Dinh-Desmarquet C, Orth G, Schiller JT, Lowy DR. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J Virol 1995;69:3959–63.
[17] Kirnbauer R, Chandrachud LM, O'Neil BW, Wagner ER, Grindlay GJ, Armstrong A, McGarvie GM, Schiller JT, Lowy DR, Campo MS. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology 1996;219:37–44.
[18] Rose RC, Lane C, Wilson S, Suzich JA, Rybicki E, Williamson AL. Oral vaccination of mice with human papillomavirus virus-like particles induces systemic virus-neutralizing antibodies. Vaccine 1999;17:2129–35.
[19] Ball JM, Hardy ME, Atmar RL, Conner ME, Estes MK. Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. J Virol 1998;72:1345–53.
[20] Estes MK, Ball JM, Guerrero RA, Opekun AR, Gilger MA, Pacheco SS, Graham DY. Norwalk virus vaccines: challenges and progress. J Infect Dis 2000;181:S367–73.
[21] Li T-C, Zhang J, Shinzawa H, Ishibashi M, Sata M, Mast EE, Kim JP, Miyamura T, Takeda N. An Empty Virus-like Particle-based Enzyme-linked Immunosorbent Assay for Antibodies to Hepatitis E Virus, J. Med. Virol., in press
[22] Tsarev SA, Tsareva TS, Emerson SU, Govindarajan S, Shapiro M, Gerin JL, Purcell RH. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proc Natl Acad Sci USA 1994;91:10198–202.
[23] Ball JM, Graham DY, Opekun AR, Gilger MA, Guerrero RA, Estes MK. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. Gastroenterology 1999;117:40–8.
[24] Clements JD, Finkelstein RA. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect Immun 1979;24:760–9.
[25] Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 1988;6:269–77.
[26] Dickinson BL, Clements JD. Use of *Escherichia coli* heat-labile enterotoxin as an oral adjuvant. In: Kiyono H, Ogra PL, McGhee JR, editors. Mucosal Vaccines. San Diego, CA: Academic Press, 1996:73–87.
[27] Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. Proc Natl Acad Sci USA 1992;89:421–5.
[28] Bockman DE, Cooper MD. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and

Peyer's patches. An electron microscopic study. Am J Anat 1973;136:455–77.
[29] Inman LR, Cantey JR. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. J Clin Invest 1983;71:1–8.
[30] Neutra MR. Interactions of viruses and microparticles with apical plasma membranes of M cells: implications for human immunodeficiency virus transmission. J Infect Dis 1999;179:S441–3.
[31] Owen RL. Uptake and transport of intestinal macromolecules and microorganisms by M cells in Peyer's patches — a personal and historical perspective. Semin Immunol 1999;11:157–63.
[32] Wolf JL, Rubin DH, Finberg R, Kauffman RS, Sharpe AH, Trier JS, Fields BN. Intestinal M cells: a pathway for entry of reovirus into the host. Science 1981;212:471–2.
[33] Elson CO. Induction and control of the gastrointestinal immune system. Scand J Gastroenterol Suppl 1985;114:1–15.
[34] Leishman AJ, Garside P, Mowat AM. Induction of oral tolerance in the primed immune system: influence of antigen persistence and adjuvant form. Cell Immunol 2000;202:71–8.
[35] Smith KM, Eaton AD, Finlayson LM, Garside P. Oral tolerance. Am J Respir Crit Care Med 2000;162:S175–8.

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2000, p. 3492–3494 Vol. 38, No. 9
0095-1137/00/$04.00+0

Molecular Cloning, Expression, and Antigenicity of Seto Virus Belonging to Genogroup I Norwalk-Like Viruses

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Received 7 January 2000/Returned for modification 13 April 2000/Accepted 30 June 2000

The viral capsid protein of the Seto virus (SeV), a Japanese strain of genogroup I Norwalk-like viruses (NLVs), was expressed as virus-like particles using a baculovirus expression system. An antigen detection enzyme-linked immunosorbent assay based on hyperimmune antisera to recombinant SeV was highly specific to homologous SeV-like strains but not heterologous strains in stools, allowing us type-specific detection of NLVs.

Norwalk-like viruses (NLVs), one of the four genera in the family *Caliciviridae* (3), are a genetically and antigenically heterogeneous group of viruses that are a major cause of acute nonbacterial gastroenteritis (1, 13). Detection and molecular characterization of NLVs have been hampered by a lack of cell culture systems and small animal models. However, recent progress in the molecular cloning and sequencing of RNA-dependent RNA polymerase and capsid protein genes has enabled us to divide NLVs into at least two genogroups: genogroup I (GI) and genogroup II (GII) (22).

NLVs contain a single-stranded positive-sense RNA genome that contains three open reading frames (ORFs) (12, 17). When the ORF2 gene is expressed by a recombinant baculovirus, the recombinant protein spontaneously self-assembles into virus-like particles (VLPs) that are antigenically and morphologically indistinguishable from native virions (4, 5, 6, 9, 11). The VLPs have been successfully used in structural studies (19, 20) and in the development of enzyme-linked immunosorbent assays (ELISAs) for serological diagnosis of NLV infection (4, 5). Though antigen detection ELISAs using hyperimmune antisera raised against the VLPs have been developed to detect NLVs in stools (2, 6, 8), the efficiency is relatively low due to the antigenic diversity of NLVs (10). The expression of antigenically distinct VLPs and production of antisera to VLPs are needed to clarify the antigenic relationship among NLVs.

This paper describes the cDNA cloning and baculovirus expression of the viral capsid gene of the Seto virus (SeV), a member of NLV GI. In addition, we report the development and evaluation of an antigen detection ELISA based on the antisera to the recombinant capsid protein.

A stool specimen (124/89 in Table 1) from an SeV outbreak was used to clone the capsid gene. The NLV detected in this stool was designated SeV. Viral RNA was extracted from a 10% stool suspension in phosphate-buffered saline using Trizol (Gibco BRL, Gaithersburg, Md.). For cDNA synthesis, oligo(dT)$_{15}$ (Promega Co., Madison, Wis.) and Moloney murine leukemia virus reverse transcriptase (Gibco BRL) were used. A seminested PCR was performed to amplify the entire ORF2 gene. The first PCR used forward primer G1F1 (5′-TGCCCG AATTCGTAAATGAT-3′) (positions 5343 to 5362 in the Norwalk virus [NV] genome; Genbank accession no. M87661) and reverse primer G1R0 (5′-GCCATTATCGGCGCARACCAA GCC-3′) (positions 6931 to 6954), and the second PCR used forward primer G1F0 (5′-GTAAATGATGATGGCGTCTAA GGA-3′) (positions 5354 to 5377) and G1R0. An approximately 1.6-kb PCR product was cloned into TA cloning vector pCR2.1 (Invitrogen, San Diego, Calif.) to generate pCR[SeV]. Nucleotide sequence analysis of the 1.6-kb insert showed that it contained the entire ORF2 of SeV and was predicted to encode a 530-amino-acid capsid protein. A comparison of the ORF2 nucleotide sequence of SeV with those of known NLVs indicated that SeV showed the highest identity with KY89 (97%), followed by NV (89%), Chiba virus (CV) (66%), Southampton virus (62%), and Desert Shield virus (62%). SeV had lower identity with GII NLVs, including the Snow Mountain virus (52%), Hawaii virus (52%), and Mexico virus (52%). The phylogenetic analysis of the ORF2 genes of SeV and representative NLVs indicated that SeV is closest to KY89 (Fig. 1).

The ORF2 gene of SeV was isolated from pCR[SeV] by digestion with *Eco*RI and inserted into a baculovirus transfer vector, pVL1392 (Pharmingen, San Diego, Calif.), at the same *Eco*RI site, to generate pVL[SeV]. Sf9 cells (Riken Cell Bank, Tsukuba, Japan) were cotransfected with 50 ng of linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold kit; Pharmingen) and 1 μg of pVL[SeV] by the Lipofectin-mediated method. A recombinant baculovirus, designated as Ac[SeV], was selected by two rounds of plaque purification. Tn5 cells (Invitrogen) were infected with Ac[SeV] at a multiplicity of infection of 10 and harvested at 6 days postinfection (p.i.) at 26.5°C. The expression of recombinant proteins was monitored by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (16). Samples were prepared

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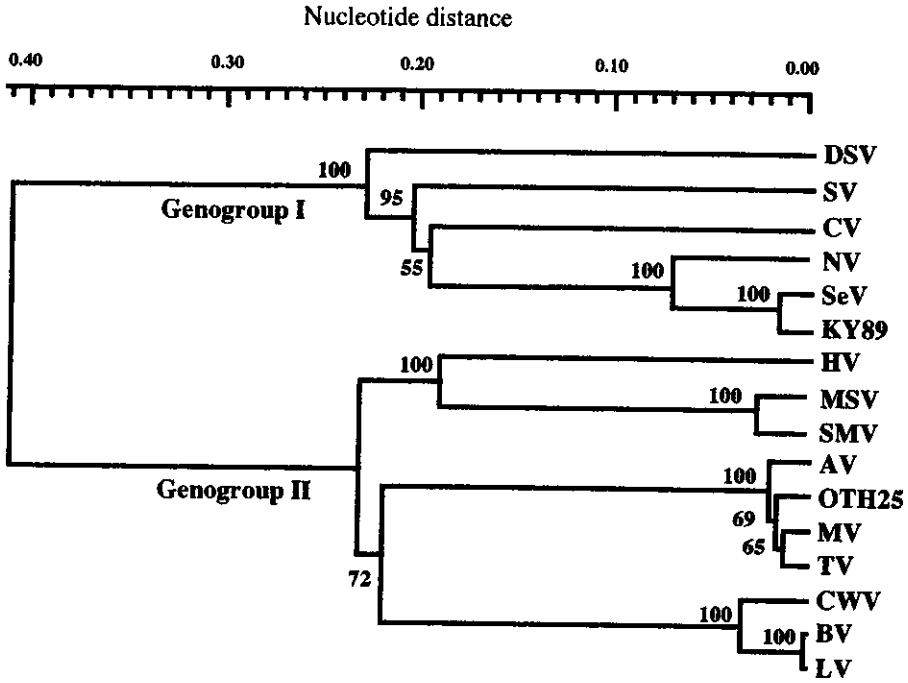

FIG. 1. Dendrogram of the ORF2 gene of SeV and known NLVs. The ORF2 genes from four GI NLVs and four GII NLVs were analyzed using a SINCA package (FUJITSU, Ltd., Tokyo, Japan), in which tree topology was inferred by UPGMA cluster analysis with the bootstrap option. The numbers at the branching points are the 50% threshold majority consensus values for 100 bootstrap replicates. The known NLV sequences (and GenBank accession numbers) are as follows: NV (M87661), KY89 (L23828), OTH25 (L23830), Southampton virus (SV) (L07418), Desert Shield virus (DSV) (U04469), CV (AB022679), Lorsdale virus (LV) (X86557), Bristol virus (BV) (X76716), Camberwell virus (CWV) (U46500), Toronto virus (TV) (U02030), Mexico virus (MV) (U22498), Snow Mountain virus (SMV) (U70059), Melksham virus (MSV) (X81879), Auckland virus (AV) (U46039), and Hawaii virus (HV) (U07611).

for electrophoresis by boiling for 3 min prior to loading. A major protein band with a molecular mass of 58 kDa was observed in the infected cells at 2 days p.i., and the expression reached its maximum at 6 days p.i. (data not shown). The observed mass of 58 kDa for the expressed protein was in agreement with the predicted molecular mass calculated from the 530-amino-acid sequence encoded by the SeV ORF2. The supernatant of the infected cells at 6 days p.i. was clarified at 10,000 × *g* for 30 min and centrifuged at 100,000 × *g* for 2 h in a Beckman TLA-45 rotor. The pellet was suspended in a few drops of water and examined by electron microscopy. Uniform, round, empty VLPs with diameters of 38 nm were observed at a concentration of 200 particles per electron micrograph field (data not shown). A typical yield of the VLPs was 0.1 to 0.2 mg per 2×10^7 Tn5 cells after CsCl equilibrium gradient centrifugation followed by sucrose density gradient centrifugation (9).

Hyperimmune antisera to the purified recombinant SeV (rSeV) were prepared in rabbits (four doses of 250 μg of protein/dose with Freund's complete adjuvant). The specificity of rabbit hyperimmune antisera to rSeV or rCV (14, 15) was tested in parallel with indirect ELISAs. The ELISA method employed was identical to the ELISA for rNV (2, 11). ELISA titers were expressed as the reciprocal of the highest dilution of antiserum giving an optical density (OD) at 492 nm of >0.15. The titers of anti-rSeV hyperimmune sera to homologous antigen were fourfold higher than those of sera to heterologous rCV (1:4,096,000 versus 1:1,024,000). The titers of anti-rCV hyperimmune sera to homologous antigen were 32-fold higher than those of sera to heterologous rSeV (1:8,192,000 versus 1:256,000). Relatively high cross-reactivity was not unexpected because broad reactivity, especially between strains included in the same genogroup, has been reported (7, 18).

An antigen detection ELISA was developed using the rabbit hyperimmune antisera to rSeV. Microplates were coated with the rabbit preimmune or hyperimmune sera (1:5,000 dilution) to capture the antigen in the stool specimens, and peroxidase-conjugated antiserum to rSeV was used as the detector antibody. The sample was considered positive when the difference between the OD values for hyperimmune and preimmune sera was >0.15 and the ratio of the hyperimmune OD to preimmune OD was >2 (15). In control experiments, hyperimmune antisera to rSeV and rCV efficiently captured 0.2 ng of the homologous antigen but did not capture the heterologous antigen (Table 1). Preimmune sera captured neither the homologous nor heterologous VLPs at any concentration. A panel of 15 stool specimens collected from patients during two SeV-associated outbreaks and a CV-associated outbreak, which had been characterized by reverse transcriptase PCR (RT-PCR) and Southern hybridization, was tested in parallel with the SeV and CV antigen detection ELISAs. In SeV-associated outbreaks, six of seven specimens (all except 125/89) were positive by RT-PCR and Southern hybridization using an SeV-specific biotinylated probe, which was prepared by PCR using pCR[SeV] as a template, as previously described (21). The probe was specific for the SeV-like strains, but not for other GI or GII NLVs (data not shown). In the CV-associated outbreak, CV-like strains from eight stool specimens were confirmed by Southern hybridization with a CV-specific probe. The antigen-detection ELISA for SeV recognized the viral antigens in stool specimens in the SeV outbreaks, but these samples were negative for the CV assay. In contrast, the CV assay detected viral

TABLE 1. Detection of NLVs in stools by ELISAs using hyperimmune antisera to rSEV and rCV

Sample	Result of antigen detection ELISA for[b]:		Result of RT-PCR and Southern hybridization
	SeV	CV	
rSEV[a]	**1.05**	0.00	NT[c]
rCV[a]	0.00	**0.93**	NT
121/89	**1.31**	0.01	+
122/89	**1.61**	0.00	+
124/89	**0.53**	0.00	+
125/89	**0.23**	0.00	−
131/89	**1.48**	0.01	+
1381/93	**0.35**	0.04	+
1382/93	**1.55**	0.04	+
663/99	0.03	**0.62**	+
665/99	0.04	**1.32**	+
666/99	0.07	**2.01**	+
669/99	0.04	**0.51**	+
675/99	0.01	**0.28**	+
676/99	0.07	**1.72**	+
680/99	0.05	**1.62**	+
687/99	0.06	**1.04**	+

[a] Purified VLPs (2 ng/ml) were used as antigens.
[b] OD values at 492 nm. Homologous titers are shown in boldface.
[c] NT, not tested.

antigens in a CV-associated outbreak but not in the SeV-associated outbreaks. SeV and CV can be differentiated by antigen detection ELISAs, although the two viruses belong to the same genogroup.

SeV showed 97% nucleotide identity (97% amino acid identity) in the capsid region with KY89 and 87% nucleotide identity (98% amino acid identity) with NV, indicating that SeV is nearly identical to KY89. SeV and KY89 were isolated in 1989 in Japan, while NV was isolated in 1968 in the United States. Although NV and KY89 have not been tested by our antigen detection ELISA, we predict that these three viruses are antigenically related.

The hyperimmune antisera to rSeV and rCV revealed minor cross-reactivities to each other when VLP antigens from cell culture were used to coat the plate to capture antibodies. In contrast, the antigen detection ELISAs, using the antibodies to coat the plate to capture antigens, were highly specific. Similarly high specificities have also been shown in the ELISAs for the detection of NV, Mexico virus, and Grimsby virus (2, 6, 8). The expression of more VLPs representing different antigenic types of NLVs, as well as the subsequent development of ELISAs based on the expressed VLPs, is necessary for the diagnosis and antigenic classification of NLVs.

Nucleotide sequence accession number. The nucleotide sequence data of SeV has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB031013.

This work was supported in part by Health Sciences Research Grants for Research on Emerging and Reemerging Infectious Diseases, Research on Environmental Health, Research on Pharmaceutical and Medical Safety, and Research on Health Sciences Focusing on Drug Innovation from the Ministry of Health and Welfare, Japan. We are grateful for the support of the Interministerial Cooperative Basic Research Core System from the Agency of Science and Technology, Japan.

REFERENCES

1. **Estes, M. K., R. L. Atmar, and M. E. Hardy.** 1997. Norwalk and related diarrhea viruses, p. 1073–1095. *In* D. D. Richmann, R. J. Whitley, and F. G. Hayden (ed.), Clinical virology. Churchill Livingstone Inc., New York, N.Y.
2. **Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M. K. Estes.** 1994. Norwalk virus infection of volunteers: new insights based on improved assays. J. Infect. Dis. **170**:34–43.
3. **Green, K. Y., T. Ando, M. S. Balayan, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H. J. Thiel.** 2000. Caliciviridae. *In* C. F. M. van Regenmortel, D. Bishop, E. Carsten, M. K. Estes, S. Lemon, J. Maniloff, M. Maya, D. McGeoch, C. R. Pringle, and R. Wickner (ed.), Virus taxonomy, in press. Academic Press, Inc., Orlando, Fla.
4. **Green, K. Y., A. Z. Kapikian, J. Valdesuso, S. Sosnovtsev, J. J. Treanor, and J. F. Lew.** 1997. Expression and self-assembly of recombinant capsid protein from the antigenically distinct Hawaii human calicivirus. J. Clin. Microbiol. **35**:1909–1914.
5. **Green, K. Y., J. F. Lew, X. Jiang, A. Z. Kapikian, and M. K. Estes.** 1993. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. J. Clin. Microbiol. **31**:2185–2191.
6. **Hale, A. D., S. E. Crawford, M. Ciarlet, J. Green, C. Gallimore, D. W. G. Brown, X. Jiang, and M. K. Estes.** 1999. Expression and self-assembly of Grimsby virus: antigenic distinction from Norwalk and Mexico viruses. Clin. Diagn. Lab. Immunol. **6**:142–145.
7. **Hale, A. D., D. C. Lewis, X. Jiang, and D. W. Brown.** 1998. Homotypic and heterotypic IgG and IgM antibody responses in adults infected with small round structured viruses. J. Med. Virol. **54**:305–312.
8. **Jiang, X., D. Cubitt, J. Hu, X. Dai, J. Treanor, D. O. Matson, and L. K. Pickering.** 1995. Development of an ELISA to detect MX virus, a human calicivirus in the Snow Mountain agent genogroup. J. Gen. Virol. **76**:2739–2747.
9. **Jiang, X., D. O. Matson, G. M. Ruiz-Palacios, J. Hu, J. Treanor, and L. K. Pickering.** 1995. Expression, self-assembly, and antigenicity of a Snow Mountain agent-like calicivirus capsid protein. J. Clin. Microbiol. **33**:1452–1455.
10. **Jiang, X., J. Wang, and M. K. Estes.** 1995. Characterization of SRSVs using RT-PCR and a new antigen ELISA. Arch. Virol. **140**:363–374.
11. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. **66**:6527–6532.
12. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195**:51–61.
13. **Kapikian, A. Z., M. K. Estes, and R. M. Chanock.** 1996. Norwalk group of viruses, p. 783–810. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.
14. **Kasuga, K., M. Tokieda, M. Ohtawara, E. Utagawa, and S. Yamazaki.** 1990. Small round structured virus associated with an outbreak of acute gastroenteritis in Chiba, Japan. Jpn. J. Med. Sci. Biol. **43**:111–121.
15. **Kobayashi, S., K. Sakae, K. Natori, N. Takeda, T. Miyamura, and Y. Suzuki.** A serotype-specific antigen ELISA in the detection of Chiba viruses in stool specimens. J. Med. Virol., in press.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
17. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science **259**:516–519.
18. **Noel, J. S., T. Ando, J. P. Leite, K. Y. Green, K. E. Dingle, M. K. Estes, Y. Seto, S. S. Monroe, and R. I. Glass.** 1997. Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. J. Med. Virol. **53**:372–383.
19. **Prasad, B. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes.** 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science **286**:287–290.
20. **Prasad, B. V. V., R. Rothnagel, X. Jiang, and M. K. Estes.** 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J. Virol. **68**:5117–5125.
21. **Takeda, N., K. Sakae, M. Agboatwalla, S. Isomura, R. Hondo, and S. Inouye.** 1994. Differentiation between wild and vaccine-derived strains of poliovirus by stringent microplate hybridization of PCR products. J. Clin. Microbiol. **32**:202–204.
22. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, and M. K. Estes.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68**:5982–5990.

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2000, p. 1656–1660
0095-1137/00/$04.00+0

Vol. 38, No. 4

Identification of an Epitope Common to Genogroup 1 "Norwalk-Like Viruses"

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Received 19 October 1999/Returned for modification 24 December 1999/Accepted 31 January 2000

A panel of 10 monoclonal antibodies (MAbs) to recombinant Norwalk virus (NV) capsid protein were tested in competition enzyme-linked immunosorbent assays. Patterns of competition indicated that these MAbs recognize six to eight epitopes covering five nonoverlapping regions of the capsid protein. A single epitope, recognized by NV MAbs NV3901, NV3912, and NV2461 was found to occur in the majority of genogroup 1 (G1) but not genogroup 2 (G2) "Norwalk-like viruses" (NLVs). This observation supports the subdivision of human NLVs into two genogroups and provides an assay for the rapid identification of G1 NLVs in fecal specimens.

"Norwalk-like viruses" (NLVs) represent one of four genera within the *Caliciviridae* and are a genetically and antigenically diverse group of agents that cause acute gastroenteritis in adults and children (5, 8). Morphologically identical viruses that are genetically related to NLVs have also been identified in fecal specimens from cows (1, 3). Human caliciviruses have not yet been successfully grown in tissue cultures, which has prevented the application of classical virological methods to study these agents. However, the ability of recombinant Norwalk virus (rNV) capsid protein to spontaneously form virus-like particles (VLPs) when expressed in insect cells has exponentially increased the experimental approaches available to characterize these viruses (17). The genetic diversity of human NLVs has also been studied by the sequencing of full and partial genomes. Phylogenetic analyses reveal two major distinct clusters of NLVs, designated genogroup 1 (G1) and genogroup 2 (G2) (24). G1 NLV strains include Norwalk virus (NV), Southampton virus, Desert Shield virus, and Chiba virus (CV). G2 NLV strains include Hawaii virus, Lordsdale virus, Grimsby virus (GRV), Mexico virus (MXV), and the Snow Mountain agent (4, 9, 10, 14, 15, 16, 18–20, 23).

NV VLPs are morphologically and antigenically similar to native virus, and their atomic structure has been solved by X-ray crystallography (21). These 38-nm particles exhibit a T=3 icosahedral symmetry, and 90 dimers of the single capsid protein form distinctive arch-like structures. The structure has a shell domain, consisting of the N-terminal 225 residues of the 530-amino-acid capsid protein, and a protruding (P) domain (22). The central region of the sequence forms the topmost P2 domain of the arch-like structures, and the C terminus forms the P1 domain which connects the shell and P2 domains, comprising the body of the arch-like structures.

The process of antigenic mapping of NV began by the generation of 10 monoclonal antibodies (MAbs) to rNV VLPs (11). The MAbs reacted by Western blotting or immunoprecipitation with either the 58K full-length capsid protein or the C-terminal 32K product produced by trypsin cleavage of soluble capsid protein and were classified into three reactivity groups: group I, consisting of four MAbs (NV834, NV142, NV101, and NV813) that recognize discontinuous epitopes on the rNV capsid protein; group II, consisting of MAbs NV3901, NV3912, and NV2461 that recognize continuous epitopes on the C-terminal 74 amino acids of the capsid protein; and group III, consisting of three MAbs (NV7411, NV8812, and NV8301) that recognize discontinuous epitopes in the C terminus of the capsid protein (11, 12). The three group II MAbs reacted with the 32K form by Western blotting even when the protein was denatured by boiling prior to electrophoresis, whereas group I MAbs NV101 and NV813 and group III MAbs only recognized the nondenatured 32K capsid protein (11). Group I MAbs NV834 and NV142 could not be mapped to the C-terminal region (11). However, all 10 MAbs detected NV in the stools of NV-infected volunteers by an enzyme-linked immunosorbent assay (ELISA) (11, 15). None of these MAbs reacted by ELISA or Western blotting with recombinant G2 MXV VLPs (11, 15).

To further characterize the epitopes recognized by these MAbs, competition ELISAs were performed using antibodies purified on protein A or G columns (Pierce, Rockford, Ill.) as previously described (2, 13). One MAb was used to coat flat-bottomed polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) overnight at 4°C at a concentration of 2 μg/ml in 0.05 M carbonate bicarbonate buffer (pH 9.6). In separate tubes, rNV VLPs, at a concentration of 5 to 500 ng/ml (depending on the coating MAb), were added to decreasing concentrations of competitor MAb (5, 1, 0.5, 0.1, and 0.05 mg/ml) in phosphate-buffered saline (PBS) (pH 7.2) containing 1% (wt/vol) BLOTTO (Carnation natural nonfat milk) and then incubated overnight at 4°C. A control of rNV in 1% BLOTTO without competitor MAb was also included in each plate. The antibody-coated microtiter plates were washed twice with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% BLOTTO for at least 30 min at 37°C. Following two additional washes with PBS-T, 60 μl of each of the rNV-MAb reaction mixtures was added to duplicate wells, and

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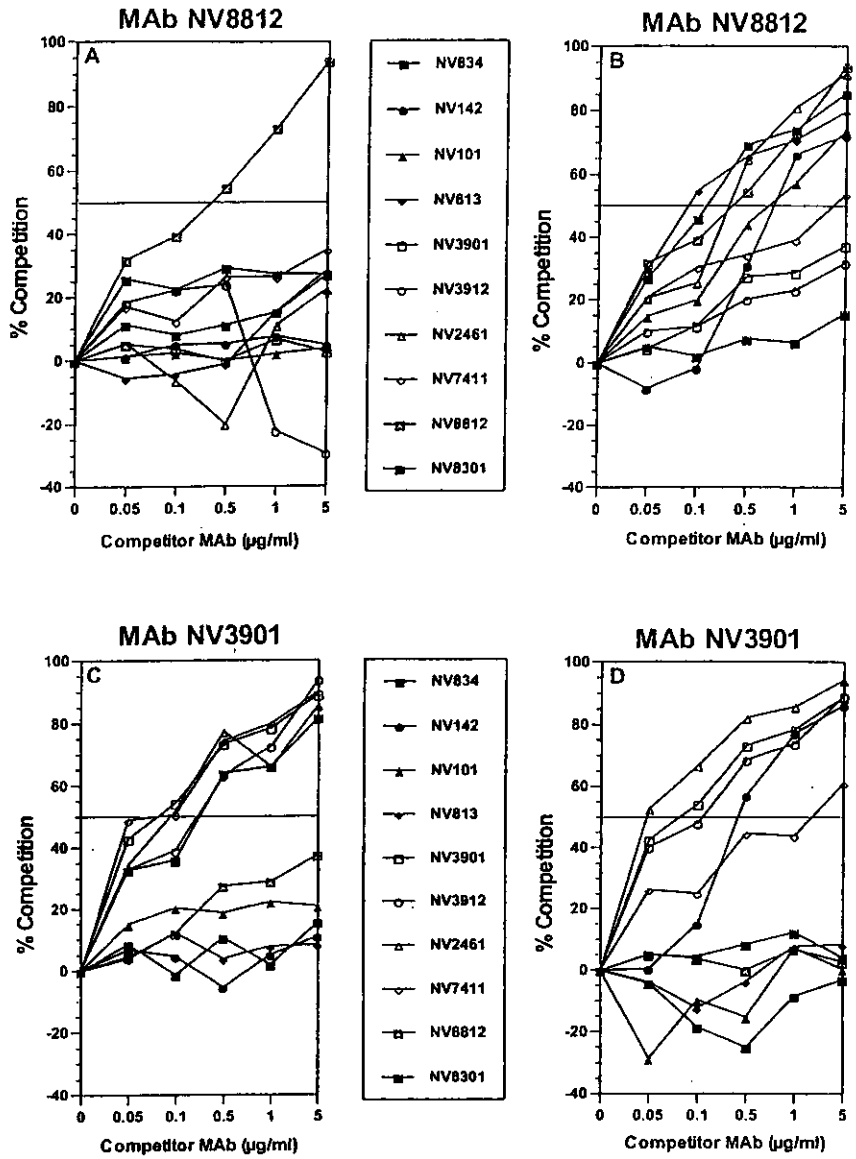

FIG. 1. Competition ELISAs showing homotypic and heterotypic competition for MAb NV8812 (top) used either as coating antibody (A) or competitor antibody (B) or MAb NV3901 (bottom) either as coating antibody (C) or as competitor antibody (D). The 50% cutoff for significant competition is indicated by a horizontal line.

the plates were incubated for 2 h at 37°C. After washing four times, 100 μl of a 1:5,000 dilution of rabbit anti-rNV hyperimmune serum in 1% BLOTTO was added to all wells. Plates were again incubated for 2 h at 37°C and washed four times, and a 1:5,000 dilution of goat anti-rabbit total immunoglobulins (Igs) (IgM, IgG, and IgA) conjugated to horseradish peroxidase (Cappel, West Chester, Pa.) was added. After a final incubation of 1 h at 37°C and four washes with PBS-T, 100 μl of 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added, and the color reaction was stopped by the addition of 100 μl of 1 M phosphoric acid. The optical density at 450 nm (OD_{450}) of wells was read, and the average of duplicates was calculated. The percentage of competition or enhanced binding was determined for all competitor MAb concentrations based on the value of the PBS control (i.e., the value of rNV binding to coating MAb in the absence of competitor MAb was defined as zero). Homotypic competition was included as a positive control for all coating MAbs.

MAbs NV3901, NV3912, and NV2461 recognize a common epitope on rNV VLPs. Initial experiments revealed that MAbs bound to the solid phase had different affinities for rNV capsid protein (data not shown). Thus, for the competition assays, the concentration of rNV VLPs used for each coating MAb was that which produced an OD_{450} of 0.5 to 1.5 when no competitor MAb was present. In agreement with other studies (2, 13), homotypic competition for the 10 MAbs was found to vary between 50 and 90% at the highest concentration of competitor antibody used in the ELISAs (data not shown). Significant competition was defined as at least a 50% reduction in detection signal at a concentration of 5 μg/ml of competitor antibody and when a concentration-dependent competition effect was observed.

No significant enhanced binding was observed, and the de-

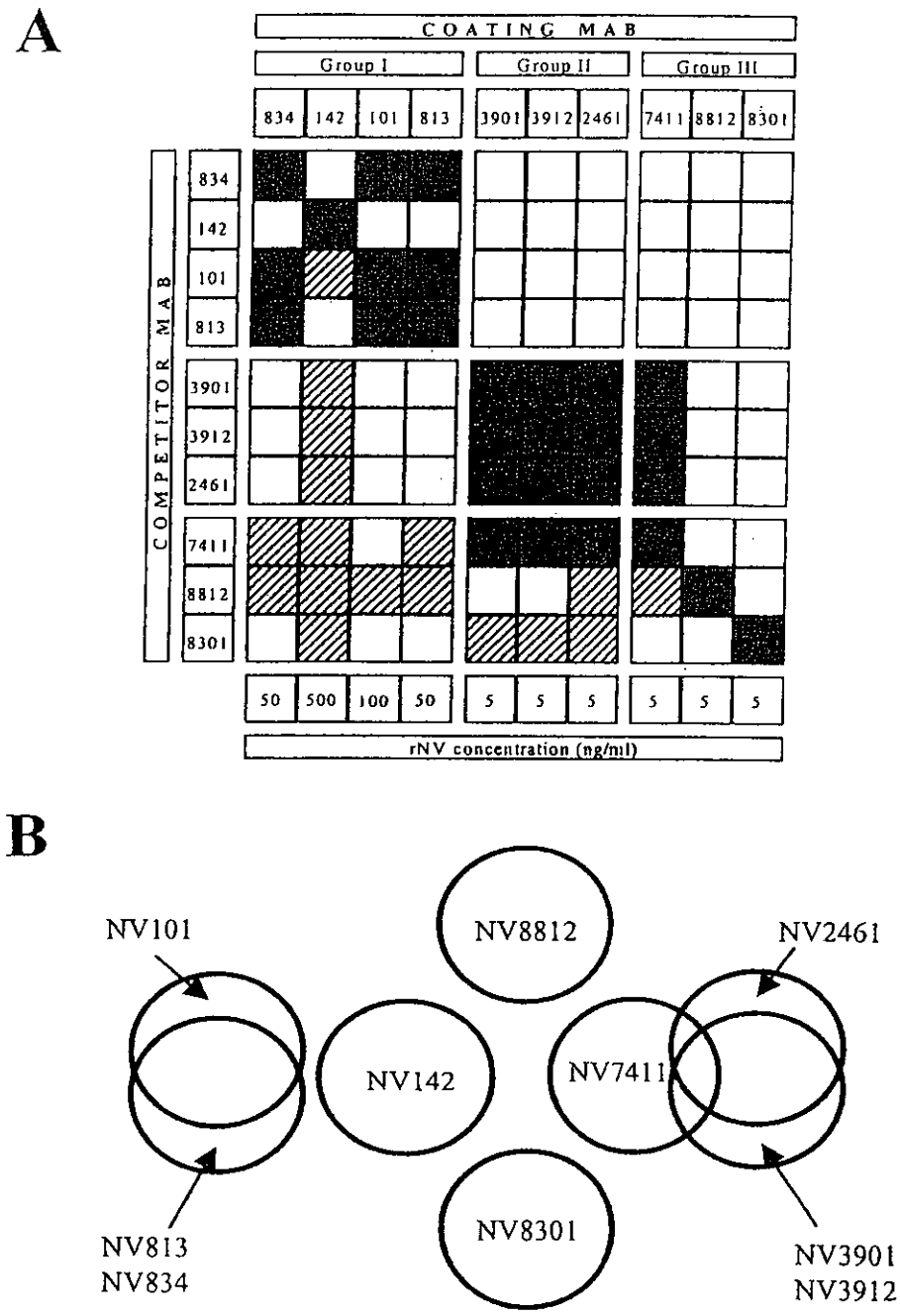

FIG. 2. (A) Summary of epitope-mapping data. Pattern of competition of 10 anti-rNV MAbs, both as coating and competitor antibodies (as indicated). Combinations producing one-way competition are indicated by hatched lines and those producing two-way competition are indicated by black shading. MAbs are grouped according to Hardy et al. (11), and the concentration (5 to 500 ng/ml) of rNV VLPs used for each coating MAb is indicated at the bottom. (B) Proposed map of the putative six to eight epitopes on rNV VLPs defined by 10 rNV MAbs.

gree of heterotypic competition was found to vary from zero to over 90% (Fig. 1). The pattern of competition differed depending on whether a MAb was used as a coating or as a competitor antibody. When group III MAb NV8812 was used as the coating MAb, only homotypic competition was observed (Fig. 1A). However, when MAb NV8812 was used as the competitor antibody, MAb NV8812 competed with itself and with six additional MAbs (NV2461, NV834, NV813, NV101, NV142, and NV7411) (Fig. 1B). One-way competition was also observed when group I MAb NV142 was used as the coating MAb for all other MAbs except group I MAbs NV813 and NV834 (data not shown). Therefore, group I MAb NV142 is unique when compared to the other group I MAbs. This phenomenon may be due to a conformational change in the epitope recognized by the coating antibody, leading to a reduced affinity of binding. Alternatively, the epitopes may overlap, or one MAb may cause steric hindrance while the other may not. For some MAb combinations, two-way competition was observed. For example, group II MAb NV3901 competed with itself, group II MAbs NV3912 and NV2461, and group III MAb NV7411 (Fig.

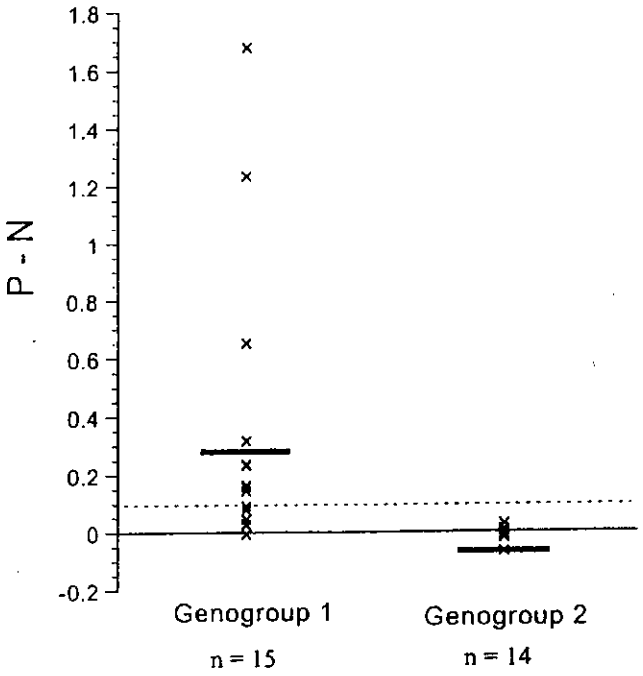

FIG. 3. Reactivity of 29 fecal specimens containing either G1 (n = 15) or G2 NLVs (n = 14) in an antigen ELISA based on MAb NV3901. The OD_{450} of MAb NV3901 (P) minus the OD_{450} of control rotavirus-specific MAb 3D8 (N) (2) is plotted for NLV G1 and G2. The cut off for reactivity (P − N >0.1) is indicated by a dashed line. Means are shown as horizontal bars.

1C and D). Group III MAb NV7411 showed a competition pattern similar to group II MAbs with the exception of the additional one-way competition of NV7411 with group I MAbs NV834 and NV813 (data not shown).

The results of the competition ELISAs for all MAb combinations are summarized in Fig. 2A. The patterns of competition were indistinguishable for a number of MAbs. Results obtained with group II MAbs NV3901 and NV3912 or MAb NV2461 were identical or similar, respectively (data not shown). Thus, group II MAbs likely recognize the same epitope or closely overlapping epitopes. Results with group I MAbs NV813, NV834, and NV101 were also identical except for the binding of group I MAb NV101 that was inhibited when group III MAb NV7411 was used as competitor antibody (data not shown). Since MAbs belonging to groups I and III recognize at least five discontinuous epitopes and two closely related discontinuous epitopes, the precise relationship of these MAbs to one another and to those in group II awaits further mapping on the rNV capsid protein. A schematic representation of the putative six to eight epitopes recognized by the 10 rNV MAbs is shown in Fig. 2B. The proposed map is compatible with the three groups described by Hardy et al. (11). In addition, group III MAb NV8812 was the only MAb to block binding of rNV to human intestinal epithelial CaCo-2 cells (25); in the present study, this MAb appears to recognize a unique epitope.

MAbs NV3901, NV3912, and NV2461 recognize a common epitope in G1 NLVs. All MAbs were tested against recombinant capsid proteins of NV, CV, GRV, and MXV by indirect ELISA as described (17), with the exception that the plates were coated with antigen at a concentration of 1 μg/ml. All MAbs reacted with rNV VLPs but did not react with recombinant MXV or recombinant GRV (11, 15; data not shown). In addition, MAbs NV3901, NV3912, and NV2461 reacted with recombinant CV VLPs, a G1 NLV exhibiting 75% amino acid sequence identity over the entire capsid with NV, 66% amino acid sequence identity over the region spanning amino acids 228 to 530 (the 32K soluble protein), and 82% amino acid sequence identity over the region spanning amino acids 457 to 530 (the C-terminal 74 amino acids containing the common epitope for G1 NLVs) (11; data not shown). The fact that these MAbs were able to detect a common epitope present on the G1 CV was both unexpected and surprising because the NV polyclonal antigen capture ELISA that uses polyclonal antibodies to NV as capture and detection antibodies does not recognize CV. To determine whether MAb NV3901, NV3912, or NV2461 could be used to detect additional G1 NLVs, an antigen capture ELISA was developed essentially as described for the NV polyclonal antigen capture ELISA (6, 17) with minor modifications. Briefly, purified MAb NV3901 or rotavirus-specific MAb 3D8 (2) was used to coat flat-bottomed polyvinylchloride microtiter plates (Dynatech Laboratories, Inc.) at a concentration of 2 μg/ml in 0.05 M carbonate bicarbonate buffer (pH 9.6). A 1:5,000 dilution of guinea pig hyperimmune serum prepared against rNV VLPs (6, 17) in 1% BLOTTO was used as the detector antibody.

A panel of 29 fecal specimens containing G1 (n = 15) or G2 (n = 14) NLVs that had been characterized by reverse transcription-PCR and amplicon sequencing (7) were tested in the NV polyclonal and MAb NV3901 antigen capture ELISAs. None of the 29 fecal specimens reacted in the NV polyclonal antigen capture ELISA (data not shown), and the P − N values of the 29 fecal specimens in the MAb NV3901 ELISA are shown in Fig. 3. Nine of 15 (60%) fecal specimens containing strains from the G1 NLV genetic clusters (7) Musgrove, Southampton, Desert Shield, and Queens Arms (but not Winchester) were detected in the ELISA with MAb NV3901 (Table 1). None of the fecal specimens containing G2 NLV strains, represented by the Hawaii virus, Lordsdale virus, MXV, or Leeds G2 NLV genetic clusters (7), reacted in the MAb

TABLE 1. Reactivity of 29 fecal specimens containing NLVs with the MAb NV3901 antigen ELISA

Outbreak strain	Genogroup (designation)[a]	No. tested	No. positive
NLV/Win/94/UK	G1 (Winchester)	2	0
NLV/Hay/89/UK	G1 (Musgrove)	1	1
NLV/But/96/UK	G1 (Musgrove)	3	2
NLV/WR/96/UK	G1 (Southampton)	2	1
NLV/B291/93/UK	G1 (Desert Shield)	1	1
NLV/L&G/96/UK	G1 (Desert Shield)	4	3
NLV/QA/92/UK	G1 (Queens Arms)	1	0
NLV/Val/95/Malta	G1 (Queens Arms)	1	1
NLV/WGH/96/UK	G2 (Hawaii)	2	0
NLV/Elm/93/UK	G2 (Hawaii)	1	0
NLV/Grl/93/UK	G2 (Hawaii)	1	0
NLV/RBH/93/UK	G2 (Mexico)	2	0
NLV/RHCH/96/UK	G2 (Lordsdale)	2	0
NLV/ELH/96/UK	G2 (Lordsdale)	1	0
NLV/Bai/96/UK	G2 (Lordsdale)	1	0
NLV/Tru/96/UK	G2 (Lordsdale)	1	0
NLV/B288/93/UK	G2 (Lordsdale)	1	0
NLV/Col/93/UK	G2 (Leeds)	2	0
Total G1 NLVs		15	9
Total G2 NLVs		14	0
Total NLVs		29	9

[a] According to Green et al. (7).

NV3901 ELISA (Table 1). The amino acid sequence identities, corresponding to the entire 539, 542, 544, 545, or 547 amino acids of the capsid protein of the G1 NLV strains tested, ranged from 63 to 70% when compared to the corresponding 530 amino acids of the capsid protein of NV (7). The level of amino acid sequence identity between NV and the G1 NLVs over the region spanning the C-terminal 74 amino acids of the capsid protein ranged from 79 to 90%. The two strains from the Winchester G1 NLV genetic cluster failed to react in the MAb NV3901 ELISA, possibly because they share the lowest capsid protein (63%) or C-terminal (79%) amino acid identity with NV. The amino acid identities of the different G2 NLV strains varied from 40 to 43% when compared to the corresponding capsid region of NV (7). Therefore, the ability of the MAb NV3901 antigen capture ELISA to detect a subset of G1 NLVs may possibly reflect the greater sensitivity of PCR over antigen detection by ELISA, differences in virus concentration among the fecal samples tested, or the result of genetic variation among the different G1 NLV genetic clusters. The main advantage of using the MAb3901 antigen capture ELISA to identify most G1 NLV strains over molecular techniques such as PCR is that large numbers of samples could be tested in a rapid and cost-effective manner.

In conclusion, MAbs NV2461, NV3901, and NV3912 appear to map to a single epitope that is common to G1 NLVs. This common epitope is present in monomeric forms of the 32K trypsin cleavage product and has been mapped to the C-terminal 74 amino acids of the capsid (11). Studies are underway to further map this epitope. These studies may allow determination of an equivalent epitope in G2 NLVs using sequence alignments directed by the rNV capsid atomic structure (21). Antibodies directed to the putative common epitope in G2 NLVs, in combination with MAb NV3901 that recognizes the common epitope in G1 NLVs, might allow the development of a broadly cross-reactive ELISA to rapidly and efficiently identify G1 and G2 NLVs in stool samples as a cause of outbreaks and sporadic cases of gastroenteritis worldwide.

We are extremely grateful to Sue E. Crawford, Robert L. Atmar, and Pamela J. Glass for helpful discussions.

This work was supported by Public Health Service grant AI38036 from the National Institute of Allergy and Infectious Diseases. Antony D. Hale undertook this work while on a fellowship from The Pathological Society of Great Britain and Ireland.

REFERENCES

1. **Bridger, J. C., G. A. Hall, and J. F. Brown.** 1984. Characterization of a calici-like virus (Newbury agent) found in association with astrovirus in bovine diarrhea. Infect. Immun. 43:133–138.
2. **Burns, J. W., H. B. Greenberg, R. D. Shaw, and M. K. Estes.** 1988. Functional and topological analyses of epitopes on the hemagglutinin (VP4) of the simian rotavirus SA11. J. Virol. 62:2164–2172.
3. **Dastjerdi, A. M., J. Green, C. I. Gallimore, D. W. G. Brown, and J. C. Bridger.** 1999. The bovine Newbury agent-2 is genetically more closely related to human SRSVs than to animal caliciviruses. Virology 254:1–5.
4. **Dingle, K. E., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1995. Human enteric *Caliciviridae*: the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J. Gen. Virol. 76:2349–2355.
5. **Estes, M. K., R. L. Atmar, and M. E. Hardy.** 1997. Norwalk virus and other enteric caliciviruses, p. 1009–1034. *In* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract. Raven Press, Ltd., New York, N.Y.
6. **Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M. K. Estes.** 1994. Norwalk virus infection of volunteers: new insights based on improved assays. J. Infect. Dis. 170:34–43.
7. **Green, J., J. Vinjé, C. Gallimore, M. Koopmans, A. D. Hale, and D. W. G. Brown.** Capsid protein diversity among "Norwalk-like" viruses. Virus Genes, in press.
8. **Green, K. Y., T. Ando, M. S. Balayan, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H.-J. Thiel.** Caliciviridae. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carsten, M. K. Estes, S. M. Lemon, J. Maniloff, M. H. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses, in press. Academic Press, Orlando, Fla.
9. **Hale, A. D., S. E. Crawford, M. Ciarlet, J. Green, C. Gallimore, D. W. G. Brown, X. Jiang, and M. K. Estes.** 1999. Expression and self-assembly of Grimsby virus: antigenic distinction from Norwalk and Mexico viruses. Clin. Diagn. Lab. Immunol. 6:142–145.
10. **Hardy, M. E., S. F. Kramer, J. J. Treanor, and M. K. Estes.** 1997. Human calicivirus genogroup II capsid sequence diversity revealed by analyses of the prototype Snow Mountain agent. Arch. Virol. 142:1469–1479.
11. **Hardy, M. E., T. N. Tanaka, N. Kitamoto, L. J. White, J. M. Ball, X. Jiang, and M. K. Estes.** 1996. Antigenic mapping of recombinant Norwalk virus capsid protein using monoclonal antibodies. Virology 217:252–261.
12. **Hardy, M. E., L. J. White, J. M. Ball, and M. K. Estes.** 1995. Specific proteolytic cleavage of recombinant Norwalk virus capsid protein. J. Virol. 69:1693–1698.
13. **Hendry, R. M., B. F. Fernie, L. J. Anderson, E. Godfrey, and K. McIntosch.** 1985. Monoclonal capture antibody ELISA for respiratory syncytial virus; detection of individual viral antigens and determination of monoclonal antibody specificities. J. Immunol. Methods 77:247–258.
14. **Jiang, X., D. O. Matson, G. M. Ruiz-Palacios, J. Hu, J. Treanor, and L. K. Pickering.** 1995. Expression, self-assembly, and antigenicity of a Snow Mountain agent-like calicivirus capsid protein. J. Clin. Microbiol. 33:1452–1455.
15. **Jiang, X., D. O. Matson, F. R. Velázquez, J. J. Calva, W. M. Zhong, J. Hu, G. M. Ruiz-Palacios, and L. K. Pickering.** 1995. Study of Norwalk-related viruses in Mexican children. J. Med. Virol. 47:309–316.
16. **Jiang, X., M. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology 195:51–61.
17. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. 66:6527–6532.
18. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round structured (Norwalk-like) virus. Science 259:516–519.
19. **Lew, J. F., A. Z. Kapikian, X. Jiang, M. K. Estes, and K. Y. Green.** 1994. Molecular characterization and expression of the capsid protein of a Norwalk-like virus recovered from a Desert Shield troop with gastroenteritis. Virology 200:319–325.
20. **Lew, J. F., A. Z. Kapikian, J. Valdesuso, and K. Y. Green.** 1994. Molecular characterization of Hawaii virus and other Norwalk-like viruses: Evidence of genetic polymorphism among human caliciviruses. J. Infect. Dis. 170:535–542.
21. **Prasad, B. V. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes.** 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286:287–290.
22. **Prasad, B. V. V., R. Rothnagel, X. Jiang, and M. K. Estes.** 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J. Virol. 68:5117–5125.
23. **Utagawa, E. T., N. Takeda, S. Inouye, K. Kasunga, and S. Yamazaki.** 1994. 3'-Terminal sequence of a small round structured virus (SRSV) in Japan. Arch. Virol. 135:185–192.
24. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, and M. K. Estes.** 1994. Sequence diversity of small round structured viruses in the Norwalk virus group. J. Virol. 68:5982–5990.
25. **White, L. J., J. M. Ball, M. E. Hardy, T. N. Tanaka, N. Kitamoto, and M. K. Estes.** 1996. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. J. Virol. 70:6589–6597.