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Binding of Norovirus Virus-Like Particles (VLPs) to Human Intestinal Caco-2 Cells and the Suppressive Effect of Pasteurized Bovine Colostrum on This VLP Binding

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Noroviruses (NoVs), which cannot be grown in cell culture, are a major infectious agent of gastroenteritis. An *in vitro* assay system was established for the evaluation of NoV binding to enterocytes using virus-like particles (VLPs) produced in a baculovirus system expressing a NoV VP1 capsid protein. After confirmation of the purity by MS analysis, VLPs were incubated with human intestinal Caco-2 cells. NoV VLPs were detected clearly by confocal laser microscopy only on a certain population of Caco-2 cells, and were semi-quantified by immunoblotting of cell lysates. Then the suppressive effect of pasteurized bovine colostrum was analyzed on the VLP binding to Caco-2 cells by immunoblotting. The colostrum reduced VLP binding in a dose-dependent manner, at about 50% suppression with 12.5 μg of the colostral proteins. Furthermore, the colostrum contained IgG antibodies reacting to VLPs, suggesting that cross-reactive antibodies in the bovine colostrums block human NoV binding to intestinal cells.

Key words: food intoxication (poisoning); nonbacterial gastroenteritis; colostral immunoglobulin; natural antibody

Noroviruses (NoVs) are major etiological agents of human nonbacterial infectious gastroenteritis, including food intoxication.[1)] Human NoVs are non-cultivable so far in that the viruses cannot be grown and replicated in cell culture systems,[2)] resulting in limited information on the infection and replication mechanisms of human NoVs. However, development of recombinant virus-like particles (VLPs) has supplied a new experimental tool for studies of interactions between human NoVs and host cells.[3)] An approximately 60-kDa capsid protein, VP1, expressed in recombinant baculovirus systems is believed to self-assemble spontaneously into VLPs 38 nm in diameter.[4)] These VLPs have been reported to be morphologically and antigenically similar to the native virions,[5)] and are regarded as empty virus particles without a virus genome.

The molecules responsible for NoV binding to host cells at the initial infection step have been searched for using NoV VLPs. Some natural and synthesized carbohydrate chains forming human histo-blood group antigens have been found to bind to NoV VLPs *in vitro*, *e.g.*, by ELISA using anti-VLPs antibodies.[6)] On the other hand, VLP binding to host cells has been investigated using metabolically radio-labeled NoV VLPs, and human intestinal Caco-2 cells were reported to be the most remarkable in the VLP binding among several cell lines, including human intestinal epithelial and embryonic kidney cells and insect fibroblastic cells,[3)] but no microscopic images showing VLP binding to Caco-2 cells have been reported. Heparan sulfate and an unidentified 105-kDa protein of Caco-2 cells were found to be possible receptors for NoV VLPs in studies using radio-labeled NoV VLPs.[7,8)] The human intestinal Caco-2 cell line is known to differentiate morphologically and functionally in confluent culture and resembles epithelial cells of small intestine[9)] despite its colon-cancer origin.[10)]

Breast milk plays important roles in supplying not only nutrients but also immunity against environmental pathogens. Especially, a special milk, termed colostrum, produced by the mammary glands in late pregnancy, is believed to be critical for neonates to grow healthy until their development of own immune systems.[11)] Colostrum from ruminant animals, including bovine ones, is different from that of humans in that bovine colostrum contains a large amount of IgG antibodies,[12)] which are absorbed by neonates in intact form across the intestinal epithelium, because the mother's serum IgGs are not delivered to the fetal circulation *via* the placenta and umbilical cord.[13)] On the other hand, human breast milk, including colostrum, contains secretory IgA antibodies, which play important roles in preventing the coloniza-

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Abbreviations: NoV, norovirus; SaV, sapovirus; VLP, virus-like particle; P-Col, pasteurized colostrum; R-milk, raw milk; R-Col, raw colostrum; MALDI-TOF, matrix-assisted laser-desorption ionization-time of flight

tion of pathogenic bacteria at the intestinal mucus epithelium.[14] In general, the biological definition of colostrum is breast milk that is synthesized and accumulated in mammary glands during the last stage of pregnancy and secreted within 1 d of giving birth. However, in Japan, bovine milk obtained within 5 d of delivery is regarded to be colostrum as a matter of regulation without definite scientific basis, and may not be used as a food.

The first aim of the present study was to establish *in vitro* assay systems to estimate NoV binding to the intestinal epithelium using non-radio-labeled VLPs and cultured Caco-2 cells, and the second was to evaluate the potential suppressive effects of bovine colostrum on NoV binding to the intestinal epithelium using established assay methods. Immunofluorescence staining of Caco-2 cells and immunoblotting analysis of the cell lysates clearly indicated that NoV VLPs bound to Caco-2 cells, and that the detection sensitivity in the established assay was comparable to that of previously reported methods using radio-labeled VLPs. Moreover, bovine colostrum obtained from healthy lactating cows at 6 or 7 d after parturition suppressed VLP binding to Caco-2 cells and contained IgGs cross-reactive to human NoV VLPs.

Materials and Methods

Bovine colostrums. Pasteurized and dried bovine colostrums were prepared at an industrial level in the facility of Kobayashi Pharmaceuticals (Osaka, Japan). For preparation of the pasteurized colostrum (P-Col), colostrums were collected from healthy lactating cows (Holstein) at 6 or 7 d after parturition and pooled. The pooled colostrums were defatted by centrifugation, pasteurized by heating at 72 °C for 15 s (an HTST condition), and concentrated by ultrafiltration, followed by spray-drying under low heating conditions. The powdered P-Col consisted of 49.8% protein, 36.4% lactose, 1.8% fat, 7.8% minerals, and 4.2% moisture. On the other hand, in the preparation of raw milk and colostrum samples (R-Milk and R-Col respectively), milk and colostrums were collected from mid-lactation (2–4 months after parturition) and early-lactation (36–48 h after parturition) healthy Holstein cows respectively bred at a local dairy farm (the Shimizu farm, Kariya, Japan). The R-Milk and R-Col samples were defatted by centrifugation and kept frozen at −20 °C until use.

Preparation of VLPs. VLPs of NoV (Ueno-7k strain) were produced by means of a baculovirus expression system, in which a recombinant VP1 capsid protein of NoV was overexpressed in an insect cell line, High Five™ (Invitrogen), the VLPs secreted into the cell medium were collected by ultracentrifugation at 30,000 rpm using an SW32 rotor (Beckman, Fullerton, CA), and the 38-nm VLPs of native virion size were purified by ultracentrifugation in CsCl containing solution.[15] The size and purity of the VLP preparation were confirmed by electron microscopy. Sapovirus (SaV) VLPs (NK24 strain) were prepared as described previously.[16]

SDS–PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli.[17] The protein samples were boiled for 3 min in SDS–PAGE sample buffer in the presence of 2-mercaptoethanol. Protein bands in gels were detected with Coomassie Brilliant Blue R-250 (CBB) staining. In immunoblotting, the separated proteins were blotted onto a poly (vinylidene difluoride) (PVDF) membrane (Immobilon; Millipore, Bedford, MA), and were visualized using anti-NoV VLP antibody[18] using an enhanced chemiluminescence (ECL) system, as described previously.[19] The relative intensity of the immune-stained band for VP1 was analyzed semi-quantitatively by densitometric measurement using the Light Capture system and CS analyzer software version 3 (Atto Instruments, Tokyo).

Protein identification by MS analysis. The protein bands of the VLP preparation detected by CBB staining of SDS-polyacrylamide gel were excised, cut into small pieces, and subjected to the in-gel digestion. After they were destained with a 50% acetonitrile/25 mM NH_4HCO_3 mixture, the gel pieces were dehydrated with 100 µl of acetonitrile, dried by vacuum centrifugation, and then rehydrated in buffer containing 25 mM NH_4HCO_3 and 1.1 units of trypsin (Trypsin Gold, MS Grade, Promega). Following digestion for 17 h at 37 °C, the peptides were extracted from the gel stepwise with 0.1% trifluoroacetic acid, a 0.1% trifluoroacetic acid/50% acetonitrile mixture, and 100% acetonitrile. The pooled extract was concentrated by vacuum centrifugation and desalted by ZipTip µ-C18 (Millipore). The extracted peptides were subjected to MALDI (matrix-assisted laser-desorption ionization) TOF (time of flight) mass spectrometry using a 4700 Proteomics Analyzer (Applied Biosystems), as described previously.[20] The MS and MS/MS data were analyzed using Mascot software (Matrix Science, London).

Cell culture and VLP binding assay. Caco-2 cells from the American Type Culture Collection (Rockville, MD) were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO), 1% nonessential amino acids solution (Sigma), 100 U/ml of penicillin, and 100 µg/ml of streptomycin, and grown under humidified 5% CO_2 and 95% air at 37 °C. For the immunoblotting analysis, Caco-2 cells were seeded at 1.0×10^5 cells/cm^2 in 48-well culture plates (Corning, NY) and cultured for 3 d in DMEM supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2. Then the cells in each well were washed with cold DMEM and incubated with various concentrations of VLPs in DMEM at 4 °C for 1 h. After washing with cold DMEM 3 times, the cells were lysed with Laemmli buffer. After centrifugation, the supernatants were collected as cell lysates and subjected to immunoblotting analysis, as described above. African green monkey kidney cells, COS7, were cultured and treated similarly with VLPs as a negative control.

Immunofluorescence microscopy. Caco-2 cells were seeded at 1.0×10^5 cells/cm^2 onto type-I-collagen-coated coverslips in the 6-well culture plate and cultured for 3 d in DMEM supplemented with 10% FCS at 37 °C under a humidified atmosphere with 5% CO_2. Then the cells on coverslips were washed with cold PBS and incubated with various concentrations of VLPs in DMEM at 4 °C for 1 h. After washing with cold DMEM 3 times, the cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice, washed with DMEM, and blocked with NETG (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, 0.05% Triton X-100, and 0.25% gelatin). The cells were incubated with NETG containing the rabbit anti-NoV VLP for VLPs[18] or mouse anti-ZO1 (Zymed) for a cell-tight junction, and subsequently with NETG containing Alexa Fluor® 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature under darkness. The cells were stained with TOTO-3 (Molecular Probes) too for nuclear staining. After washing with PBS, the cells on coverslips were mounted onto glass slides containing a drop of 50% glycerol in PBS. Imaging was performed on a Zeiss Axioplan2 microscope equipped with a LSM5 PASCAL laser scanning confocal optics (Carl Zeiss, Thornwood, NY) in multi-track mode.

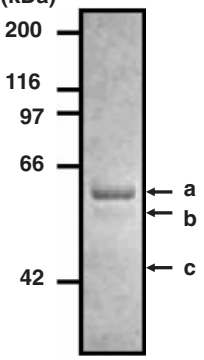
ELISA. IgG antibodies specific for NoV and SaV VLPs in bovine colostrums were measured by ELISA, as described previously.[21] Briefly, 96-well plates (Nunc, Roskilde) were coated with 1 µg per well of VLPs in PBS. After washing and blocking, the plates were incubated with colostrums diluted to a 1–20 µg protein concentration with PBS/Tween/BSA, washed with PBS/Tween, and incubated with POD-conjugated anti-bovine IgG (H and L chains) antibody (Abcam, Cambridge).

Results

NoV VLPs specifically bound to a certain population of Caco-2 cells

The NoV VLPs used in the cell-binding assay were concentrated and purified by ultracentrifugation from culture supernatants of the infected insect cells. The

A



B

Band	Protein	MSDB Accession #	Score MS	Score MS/MS	Molecular weight
a	Capsid protein - Norwalk-like virus	Q8JW42_9CALI	132	416	60709
b	Capsid protein - Norwalk-like virus	Q8JW42_9CALI	133	181	60709
c	Capsid protein - Norwalk-like virus	Q8JW42_9CALI	90	104	60709

C

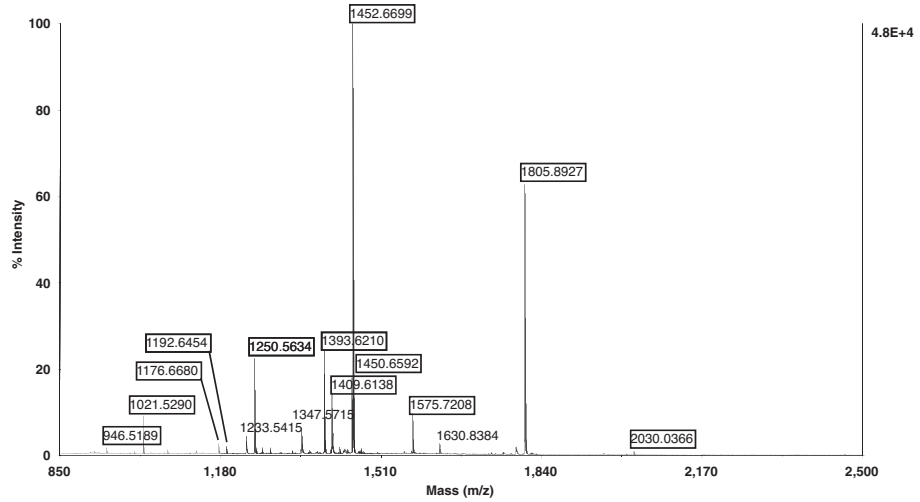


Fig. 1. SDS–PAGE and MS Analysis of NoV VLPs.

NoV VLPs (1 μg) were subjected to SDS–PAGE, followed by CBB staining (A). Each protein band (a, b, and c, indicated by arrows) was excised, in-gel digested with trypsin, and subjected to MALDI-TOF/MS analysis. All protein bands were identified as the capsid protein, VP1, of NoVs, previously termed Norwalk viruses (B) based on MS data analyzed using the Mascot software. The MS of band c is shown as a representative (C), in which mass peaks identified as VP1 are marked with open squares.

purity of the VLP preparation was examined by SDS–PAGE and subsequent MS analysis. The NoV VLP preparation gave a single major band of 60 kDa, a faint band of 57 kDa, and a nearly invisible 45-kDa band (Fig. 1A). All three protein bands were identified as intact VP1 and fragments of it by MS analysis (Fig. 1B) and their estimated molecular mass by SDS–PAGE was determined. As found in a representative mass spectrum of the 45-kDa band, most of the tryptic peptide signals were assigned to theoretical tryptic peptides of the NoV capsid protein, VP1 (Fig. 1C).

To elucidate the binding of NoV VLPs to human intestinal Caco-2 cells, an immunofluorescence microscopic approach was applied to cells incubated with the VLPs of NoV and of SaV for comparison. As shown in Fig. 2A and B, fluorescent signals of NoV VLPs were clearly detected by confocal laser scanning microscopy in several restricted cells, which formed colonies in the cell culture wells, whereas no signals of SaV VLPs were detected under the same conditions. The difference in the fluorescent signal was clear between VLP-binding positive and negative cells. Non-specific binding of the antibodies to NoV and SaV VLPs was not observed in the cells without VLP treatment. A representative three-dimensional image of a magnified field clearly indicated that NoV VLPs bound to almost the whole area of the apical cell surface of the positive cells (X/Y and Y/Z planes of Fig. 2C), whereas none did to any area of the negative cells (X/Y and X/Z planes of Fig. 2C).

The binding of NoV VLPs to Caco-2 cells was analyzed in more detail by immunoblotting of total cell lysates. Caco-2 cells and COS7 cells as a negative control were incubated with the VLPs (100 ng) for various periods of time up to 180 min. Immunostained VP1 band of Caco-2 cell lysates became detectable after 5 min of incubation, and the band intensity increased with incubation time, whereas that of COS7 lysates was faintly detected only in the sample of 180 min of incubation (Fig. 3A). Based on the band intensity obtained by densitometric analysis, the NoV VLPs bound to the Caco-2 cells in a well at 180 min of incubation was estimated to be about 16 ng (data not shown). To estimate the binding affinity of VLPs to Caco-2 cells as well as the detection sensitivity of bound VLPs by immunoblotting, various amounts (5 to 150 ng per well) of VLPs were incubated with Caco-2 cells, and were subjected to immunoblotting analysis. The VP1 band was clearly detected at levels of 75 ng or more in a dose-dependent manner, and it was still detectable when only 5 ng of VLPs was incubated with Caco-2 cells (Fig. 3B).

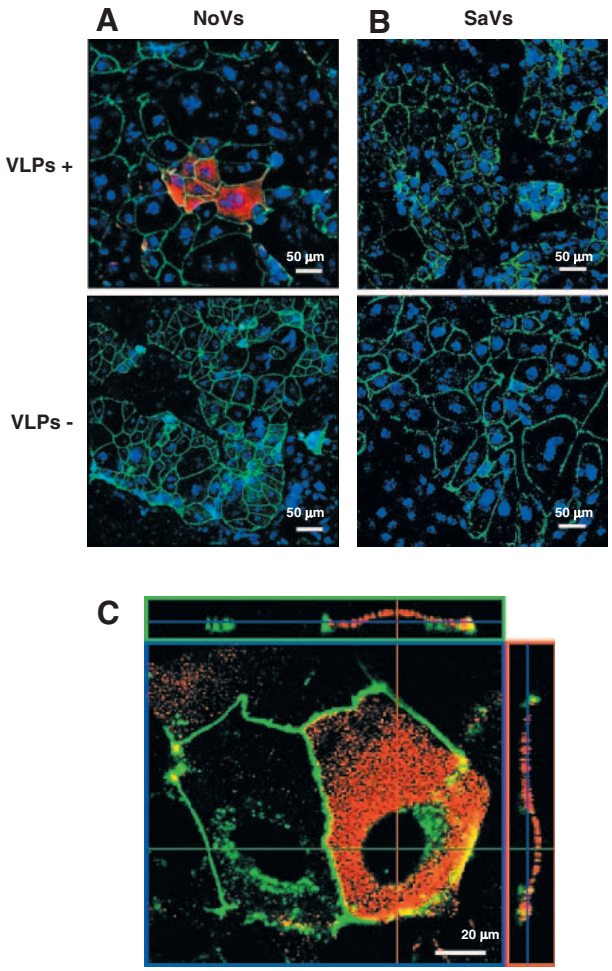


Fig. 2. Confocal Laser Scanning Microscopy of Caco-2 Cells Incubated with NoV and SaV VLPs.

Caco-2 cells were cultured for 3 d on type-I-collagen-coated coverslips in a 6-well culture plate and incubated for 1 h at 4 °C with (+) and without (−) 0.5 µg of NoV VLPs (A and C) or SaV VLPs (B). After washing and paraformaldehyde fixation, the cells were stained with rabbit anti-NoV VLP, rabbit anti-SaV VLP, and mouse anti-ZO-1 (a tight junction marker) antibodies, and subsequently with Alexa Fluor 568 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse IgG. Nuclei were stained with TOTO-3. Typical Z-axis images of Caco-2 cells incubated with NoV VLPs were constructed digitally in the XZ and YZ planes, shown as horizontal and vertical lines in the XY plane (C). Red, NoV VLPs; green, ZO-1; blue, nuclear. Bars, 50 µm in panels A and B; 20 µm in panel C.

Pasteurized bovine colostrums suppressed VLP-binding to Caco-2 cells and contained anti-NoV IgG

Utilizing the above described assay system, the suppressive effect of P-Col on NoV VLP-binding to Caco-2 cells was examined semi-quantitatively by immunoblotting. As shown in Fig. 4A, pre-incubation of the VLPs with an excess amount (25 µg protein against 0.1 µg of VLPs) of P-Col markedly decreased the band intensity of the VLPs in the Caco-2 cell lysate. Non-specific staining was not observed in either lysate of the cells incubated with the various protein concentrations of the colostrums without VLPs. The VP1 band intensity in each cell lysate was quantified by densitometric analysis (Fig. 4B), indicating that the VLPs binding to Caco-2 cells decreased gradually with increases in the amount of P-Col used in pre-incubation with VLPs, and 50% suppression was achieved with 12.5 µg of P-Col.

Bovine colostrums are known to contain large amounts of IgG. The content of the intact form of IgG consisting of two heavy and two light chains in P-Col and R-Col was estimated by SDS–PAGE under non-reduced conditions, to which various amounts (equivalent to 15–50 µg protein) of each sample were subjected (Fig. 5A). The 200-kDa band was identified as IgG by MS and immunoblotting analysis (data not shown), and it was the most dominant protein component in both R-Col and P-Col. The IgG content estimated from the band intensity was higher in P-Col than in R-Col. This higher IgG proportion of P-Col might be ascribed to the preferential concentration of IgG and other proteins during the heat pasteurization process, including the ultrafiltration described in "Materials and Methods."

Bovine IgG antibodies with reactivity to human NoV VLPs were examined by ELISA on P-Col as well as R-Col and R-Milk for comparison (Fig. 5B). Anti-NoV VLP IgG was detected remarkably not only in R-Col but also in P-Col, while R-Milk showed almost no IgG-binding activity to the VLPs. The IgG reactivity to NoV VLPs of P-Col was a little higher than that of R-Col. VLP binding activity in P-Col was analyzed more in detail at various protein-concentrations using VLPs of SaV in addition to NoV, which belong to the same Calicivirus family. As shown in Fig. 5C, the IgG antibodies in P-Col were reactive to VLPs not only of NoV but also of SaV, and the ELISA value increased in a manner dependent on the sample protein concentration. The rabbit antibody raised by immunizing with NoV VLPs did not cross-react with SaV VLPs under either ELISA or immunoblotting analysis (data not shown).

Discussion

A single VLP is believed to be constructed by spontaneous association of 180 molecules of a single capsid protein, VP1, and the structural or architectural similarity to virus virions has been demonstrated by X-ray crystallography.[22] Moreover, antibodies prepared by immunizing animals with VLPs react well with virus virions in patients' specimens, and thus immunochemical detection of etiological agents became possible in diagnosis for NoV food poisoning. Hence, the morphological and immunochemical properties of VLPs have been investigated, while the components of VLPs have not been well characterized biochemically. In the present study, the purity and intactness of VLPs were analyzed by SDS–PAGE and MS-based protein identification, in which all of one major and two minor proteins were identified to be VP1 (Fig. 1). This indicates that the VLPs prepared by the baculovirus expression system are constructed from a single capsid protein, VP1, as expected, without contaminating proteins from the expression system, but a trace amount of degraded VP1 was also present in the VLPs. Nevertheless, such a faint proteolysis of VP1 is expected not to affect the VLPs-cells binding assay, and therefore the VLP preparation was used in the binding assay. It is unlikely that the degraded VP1 or partially degraded VLPs inhibited the binding of intact VLPs to the cells.

A recent study on NoV infection using biopsy specimens from the duodenum of patients with NoV

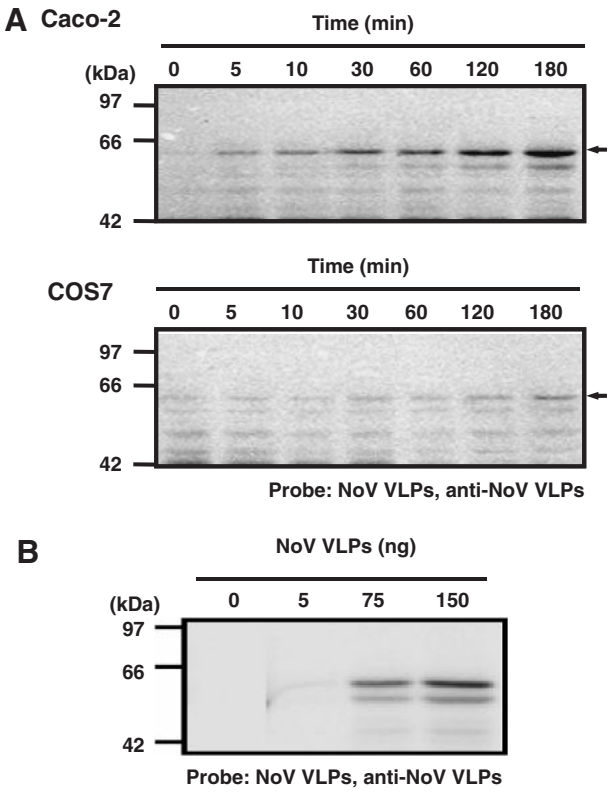


Fig. 3. Immunoblotting Analysis of NoV VLPs Bound to Caco-2 Cells.

Caco-2 cells were cultured in 48-well culture plates for 3 d and then incubated with NoV VLPs (0.1 μg per well) at 4 °C for various periods (5–180 min). Total cell lysates were subjected to SDS–PAGE, followed by immunoblotting for NoV VP1 (A). The migration position of NoV VP1 is indicated by arrows. The cells were incubated with various amounts of NoV VLPs (5, 75, and 150 ng per well) for 60 min, and NoV VLPs were analyzed by immunoblotting as above (B).

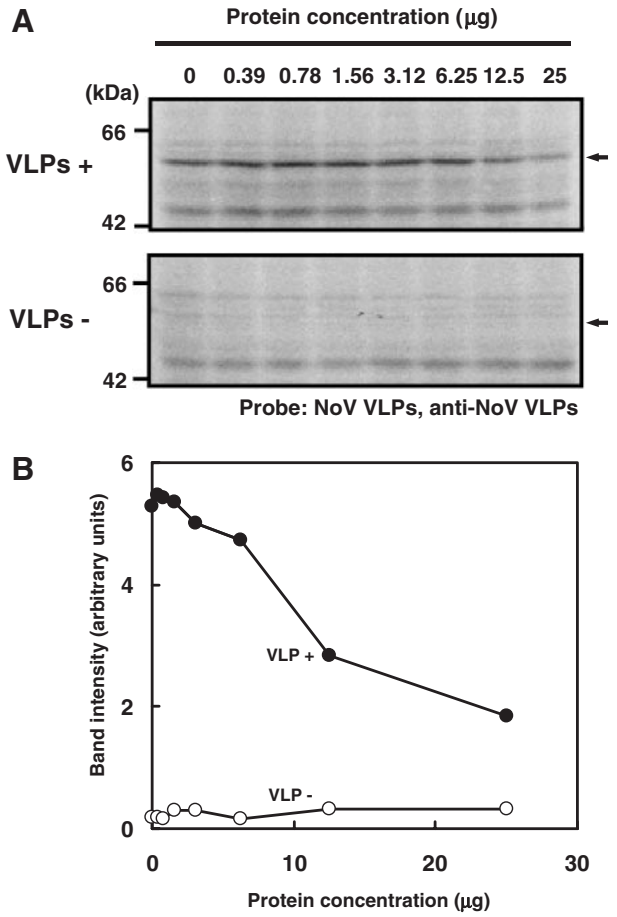


Fig. 4. Suppression of NoV VLP Binding to Caco-2 Cells by Pasteurized Bovine Colostrum.

NoV VLPs (50 ng) and a buffer control without the VLPs were pre-incubated for 15 h at 4 °C with various amounts (0.39–25 μg) of pasteurized bovine colostrum (P-Col), and then incubated with Caco-2 cells for 1 h at 4 °C, followed by immunoblotting analysis for NoV VLPs, as described in the legend to Fig. 3A. The migration position of NoV VP1 is indicated by arrows. The VP1 band intensities were digitized and semi-quantified by densitometric analysis (B). Closed and open circles represent NoV VLPs and the buffer control respectively.

infection indicated that NoV infected and replicated in the epithelial cells of the small intestine.[23] NoV infection at the distal small intestine and colon remains to be investigated. Caco-2 is a cell line established from human colon cancer cells.[10] Nevertheless, Caco-2 cells in confluent culture express several hydrolytic enzymes and nutrient transporters, typical markers of absorptive epithelial cells of the small intestine.[9] Therefore, the observation, in the present and previous studies,[3,7] that NoV VLPs bound to Caco-2 cells, especially differentiated ones, is reasonable. A recent reverse genetic study of human NoV indicated that native NoV could be produced in Caco-2 cells into which NoV genomic RNA had been transfected.[24] Thus it is likely that the NoV-binding assay system established in the present study at least in part reflects or mimics the initial step of *in vivo* NoV infection in the human intestine. This does not necessarily rule out the possibility of NoV infection in human colon epithelia.

Caco-2 cells might consist of heterogeneous cell populations, because they have not necessarily been cloned. The observation that only a small population of colonized Caco-2 cells was positive to NoV VLP binding (Fig. 2) might be explained by this authentic heterogeneity of Caco-2 cells. The frequency of the VLP-binding positive cells in cultured Caco-2 cells increased when the cells were cultured for longer periods (10 d), but the positive cells were still only a minor population (Murakami and Matsuda, unpublished results). NoV VLPs might bind preferentially to differentiated cells in a certain population of Caco-2 cells. In fact, differentiated Caco-2 cells have been reported to be 2–3 times more active in binding with radio-labeled NoV VLPs than undifferentiated cells.[3] From a practical viewpoint, however, the preparation of well-differentiated Caco-2 cells is time consuming and costly. The results of the present study indicate that Caco-2 cells incubated for 3 d were usable in VLP-binding assay based on sensitive immunodetection systems. Thus the VLP-binding assay using undifferentiated Caco-2 cells, *e.g.*, cultured for only 3 d, should have an advantage in high throughput screening of anti-virus factors in food.

NoV VLP-binding to some restricted cells in an all-or-none manner in addition to no binding of SaV VLPs (Fig. 2) indicates that the binding of NoV Ueno-7k VLPs to Caco-2 cells is specific and significant (physiologically or pathologically relevant). Furthermore, almost no binding of NoV VLPs to COS7 cells

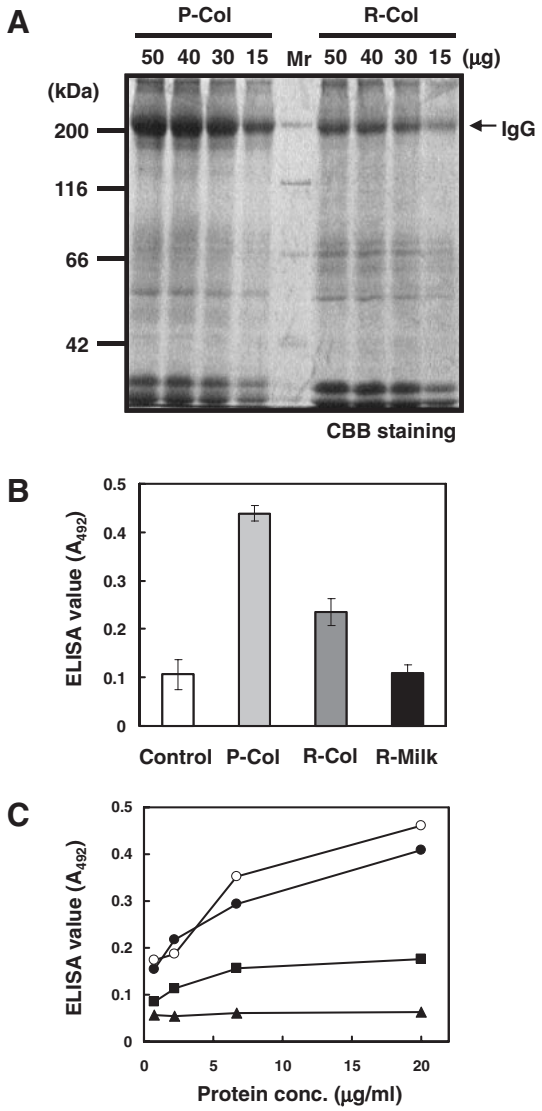


Fig. 5. Bovine Colostrum IgG with Reactivity to NoV- and SaV-VLPs.

Proteins in pasteurized and raw colostrums (P-Col and R-Col respectively) were analyzed comparatively by SDS–PAGE (10% acrylamide gel under non-reducing conditions) (A). ELISA plates were coated with NoV VLPs (0.1 μg/ml) and incubated with 10 μg of protein/ml of P-Col, R-Col, raw milk (R-Milk), and egg albumin as a control. Bovine IgGs bound to the coated VLPs were detected with POD-conjugated anti-bovine IgG (B). ELISA plates were coated with 0.1 μg/ml of NoV VLPs (solid circles and solid triangles), SaV VLPs (open circles), and gelatin (solid squares) as a control and incubated with 10 μg of protein/ml of P-Col. Bovine IgGs bound to the coated VLPs were detected with POD-conjugated anti-bovine IgG. A control assay using POD-conjugated anti-rabbit IgG was also done to test the non-specific binding of the secondary antibody (solid triangles) (C).

(Fig. 3A) suggests that NoV VLP-binding is specific to human intestinal cells. VLP-binding was detected even when only a trace amount of VLPs (5–150 ng) was incubated with Caco-2 cells (Fig. 3B). Based on these results, the binding affinity or avidity of NoV VLPs to Caco-2 cells might not be low. On the other hand, the VP1 band intensity increased almost linearly through an incubation period of 180 min, suggesting that the rate of VLP-binding is low, probably due to the low dispersion rate of VLPs much larger in size than single protein molecules. In the present study, the amounts of VLPs added or bound to the Caco-2 cells were at 10–100 nanogram levels, smaller than previously reported ones (1–10 micrograms), indicating that immunoblotting detection of VLPs using ECL systems has detection-sensitivity higher than the scintillation counting method using VLPs metabolically labeled with ^{35}S-methionine.[3)]

Because maternal IgG is not transmitted to the embryo through the placenta in ruminant species of mammals, bovine colostrums contain a large amount of IgG,[13)] which is important or critical for newborn calves to gain immunity against environmental pathogens until the development of their own immune systems. The pooled colostrums from healthy and non-immunized cows contained IgG with reactivity to human NoV VLPs, suggesting that cows' colostrums contain naturally occurring antibodies with a large repertory of antigen specificity, including human pathogens. Furthermore, colostrums are known to be rich also in antimicrobial components other than immunoglobulins, including lactoferrin, lactoperoxidase, and lysozyme.[25)] Therefore, in addition to colostral IgG, unidentified anti-NoV factors might be present in the colostrums. Hence, colostrums have been expected to contain various immunological and non-immunological defensive factors against enteric pathogens in animals, including humans, but the practical application of bovine colostrums to food as ingredients has been restricted by a Japanese regulatory issue, where bovine colostrums from day 1 to day 5 lactating cows may not be used in food, and moreover milk has to be pasteurized for the market. In the present study, bovine colostrums for P-Col were collected from lactating cows 6–7 d after parturition and heated for pasteurization under mild conditions to prevent or reduce the inactivation of antiviral factors, including antibodies and some other proteins, in the colostrums. In fact, anti-NoV VLPs IgG as well as anti-SaV VLPs IgG was detected in P-Col. Hence, the regulatory and food-safety issues would be resolved at least technically. Bovine colostrums collected at 6 d after parturition or later might be usable in food and food supplements, in which an anti-NoV effect is expected.

Another issue to be discussed is whether such active IgGs escape degradation by digestive enzymes and reach the intestine, where NoV infects the enterocytes. Secretory IgA in human milk and saliva is known to be stabilized by association with a secretory component, and to resistant against digestive enzymes.[12,26)] In ruminant animals, including cows, secretory antibodies in colostrums are IgG class,[27)] though the secretion mechanisms across the mammary gland epithelium and any structural changes or modifications during secretion remain to be investigated. Some food proteins have been reported to remain intact, at least at immunologically detectable levels, in the lumen of the mouse small intestine.[21,28)] It would be of interest to investigate the digestibility or resistance of bovine colostrum IgG *in vitro* and *in vivo* using animal models.

In conclusion, *in vitro* binding-assay using NoV VLPs with Caco-2 cells can be a useful tool in primary screening for anti-NoV in food and natural products, and bovine colostrums have some potential as an infection-protective factor against enteroviruses, including NoV and SaV.

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References

1) Griffin MR, Surowiec JJ, McCloskey DI, Capuano B, Pierzynski B, Quinn M, Wojnarski R, Parkin WE, Greenberg H, and Gary GW, *Am. J. Epidemiol.*, **115**, 178–184 (1982).
2) Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, and Estes MK, *J. Gen. Virol.*, **85**, 79–87 (2004).
3) White LJ, Ball JM, Hardy ME, Tanaka TN, Kitamoto N, and Estes MK, *J. Virol.*, **70**, 6589–6597 (1996).
4) Jiang X, Wang M, Graham DY, and Estes MK, *J. Virol.*, **66**, 6527–6532 (1992).
5) Green KY, Lew JF, Jiang X, Kapikian AZ, and Estes MK, *J. Clin. Microbiol.*, **31**, 2185–2191 (1993).
6) Huang P, Farkas T, Marionneau S, Zhong W, Ruvoen-Clouet N, Morrow AL, Altaye M, Pickering LK, Newburg DS, LePendu J, and Jiang X, *J. Infect. Dis.*, **188**, 19–31 (2003).
7) Tamura M, Natori K, Kobayashi M, Miyamura T, and Takeda N, *J. Virol.*, **78**, 3817–3826 (2004).
8) Tamura M, Natori K, Kobayashi M, Miyamura T, and Takeda N, *J. Virol.*, **74**, 11589–11597 (2000).
9) Meunier V, Bourrie M, Berger Y, and Fabre G, *Cell Biol. Toxicol.*, **11**, 187–194 (1995).
10) Fogh J, Fogh JM, and Orfeo T, *J. Natl. Cancer Inst.*, **59**, 221–226 (1977).
11) Newburg DS and Walker WA, *Pediatr. Res.*, **61**, 2–8 (2007).
12) Stelwagen K, Carpenter E, Haigh B, Hodgkinson A, and Wheeler TT, *J. Anim. Sci.*, **87**, 3–9 (2009).
13) Barrington GM and Parish SM, *Vet. Clin. North Am. Food Anim. Pract.*, **17**, 463–476 (2001).
14) Newburg DS, *J. Nutr.*, **135**, 1308–1312 (2005).
15) Hansman GS, Natori K, Shirato-Horikoshi H, Ogawa S, Oka T, Katayama K, Tanaka T, Miyoshi T, Sakae K, Kobayashi S, Shinohara M, Uchida K, Sakurai N, Shinozaki K, Okada M, Seto Y, Kamata K, Nagata N, Tanaka K, Miyamura T, and Takeda N, *J. Gen. Virol.*, **87**, 909–919 (2006).
16) Hansman GS, Natori K, Oka T, Ogawa S, Tanaka K, Nagata N, Ushijima H, Takeda N, and Katayama K, *Arch. Virol.*, **150**, 21–36 (2005).
17) Laemmli UK, *Nature*, **227**, 680–685 (1970).
18) Shirato-Horikoshi H, Ogawa S, Wakita T, Takeda N, and Hansman GS, *Arch. Virol.*, **152**, 457–461 (2007).
19) Nakatani H, Aoki N, Nakagawa Y, Jin-No S, Aoyama K, Oshima K, Ohira S, Sato C, Nadano D, and Matsuda T, *Biochem. J.*, **395**, 21–30 (2006).
20) Okumura H, Kohno Y, Iwata Y, Mori H, Aoki N, Sato C, Kitajima K, Nadano D, and Matsuda T, *Biochem. J.*, **384**, 191–199 (2004).
21) Matsubara T, Aoki N, Honjoh T, Mizumachi K, Kurisaki J, Okajima T, Nadano D, and Matsuda T, *Biosci. Biotechnol. Biochem.*, **72**, 2555–2565 (2008).
22) Venkataram Prasad BV, Hardy ME, and Estes MK, *J. Infect. Dis.*, **181** (Suppl 2), S317–S321 (2000).
23) Troeger H, Loddenkemper C, Schneider T, Schreier E, Epple HJ, Zeitz M, Fromm M, and Schulzke JD, *Gut*, **58**, 1070–1077 (2009).
24) Guix S, Asanaka M, Katayama K, Crawford SE, Neill FH, Atmar RL, and Estes MK, *J. Virol.*, **81**, 12238–12248 (2007).
25) Wheeler TT, Hodgkinson AJ, Prosser CG, and Davis SR, *J. Mammary Gland Biol. Neoplasia*, **12**, 237–247 (2007).
26) Woof JM and Kerr MA, *J. Pathol.*, **208**, 270–282 (2006).
27) Stephan W, Dichtelmuller H, and Lissner R, *J. Clin. Chem. Clin. Biochem.*, **28**, 19–23 (1990).
28) Yamada C, Yamashita Y, Seki R, Izumi H, Matsuda T, and Kato Y, *Biosci. Biotechnol. Biochem.*, **70**, 1890–1897 (2006).