Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters[∇]

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Bivalve molluscan shellfish, such as oysters, filter large volumes of water as part of their feeding activities and are able to accumulate and concentrate different types of pathogens, particularly noroviruses, from fecal human pollution. Based on our previous observation of a specific binding of the Norwalk strain (prototype norovirus genogroup I) to the oyster digestive tract through an A-like carbohydrate structure indistinguishable from human blood group A antigen and on the large diversity between strains in terms of carbohydrate-binding specificities, we evaluated the different ligands implicated in attachment to oysters tissues of strains representative of two main genogroups of human norovirus. The GI.1 and GII.4 strains differed in that the latter recognized a sialic acid-containing ligand, present in all tissues, in addition to the A-like ligand of the digestive tract shared with the GI.1 strain. Furthermore, bioaccumulation experiments using wild-type or mutant GI.1 Viruslike particles showed accumulation in hemocytes largely, but not exclusively, based on interaction with the A-like ligand. Moreover, a seasonal effect on the expression of these ligands was detected, most visibly for the GI.1 strain, with a peak in late winter and spring, a period when GI strains are regularly involved in oyster-related outbreaks. These observations may explain some of the distinct epidemiological features of strains from different genogroups.

Bivalve molluscan shellfish, such as oysters, can filter large volumes of water as part of their feeding activities and are able to accumulate and concentrate different types of pathogens from fecal human pollution. We have known for 40 years that bacteria and viruses show differences in terms of concentration, accumulation, and depuration from contaminated shellfish (34). As a consequence, the absence of virus contamination cannot reliably be deduced from failure to detect bacterial contamination. A better understanding of the virus-specific modes of shellfish contamination is needed. A number of factors, including water temperature, mucus production, the glycogen content of connective tissue, or gonadal development have been identified to influence enterovirus and phage bioaccumulation in oysters (7, 13).

Among human enteric viruses, noroviruses (NoVs) are recognized as being the leading cause of epidemics or sporadic cases of gastroenteritis in all age groups of humans (15). They are discharged in large amounts in sewage and, being very resistant to inactivation, they have been detected in wastewater treatment plant effluents and in surface waters (44). The sanitary consequences are contamination of drinking water and foods such as shellfish, leading to outbreaks among consumers (57). Improved understanding of norovirus behavior in shellfish may lead to increased sanitary quality of shellfish on the market.

Many NoV strains bind to histo-blood group antigens (HBGAs) (50). HBGAs are complex glycans present on many

cell types, including red blood cells and vascular endothelial cells, as well as on the epithelia of the gastrointestinal, urogenital, and respiratory tracts. They are synthesized from a series of precursor structures by stepwise addition of monosaccharide units via a set of glycosyltransferases (31). Evidence accumulated from volunteer studies and from analysis of outbreaks indicates that binding to these carbohydrates is required for infection (20, 25, 27, 49). Moreover, various human NoV strains that bind to HBGAs present distinct specificities for HBGAs. As a result, most strains infect only a subset of the population based on HBGA expression (26, 51). It was proposed that NoV carbohydrate-binding properties could be used to improve detection in waters and other complex samples (9).

We previously demonstrated specific binding of the Norwalk virus strain to the oyster digestive tract through an A-like carbohydrate structure indistinguishable from human blood group A antigen (23). Subsequently, this observation was confirmed in different oyster species and for other NoV strains (52, 54). Human A blood group antigen is one of the HBGA ligands of NoVs that are involved in the infection process (26, 48), suggesting that oysters may have the ability to specifically accumulate and concentrate a human pathogen based on the presence of a shared ligand between the two species rather than through nonspecific interactions only. Since different NoV strains show different specificities for HBGAs in humans, all strains may not be captured equally well by oysters.

This new concept where the relationship between the shellfish carbohydrate ligands and the virus strain specificity is taken into account may be used as a tool to discriminate within different viruses, bivalve molluscan shellfishes, and seasons both in terms of risk analysis and for shellfish producers. The

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objectives of the present study were to develop an enzyme-linked immunosorbent assay (ELISA) approach for virus binding quantification of two NoV strains representative of the two main genogroups (GI.1 and GII.4) to various oysters tissues, to characterize the ligands involved in binding, to evaluate their relationship with bioaccumulation, and finally to assess the influence of seasonal variations on virus binding.

MATERIALS AND METHODS

Reagents and viruslike particles (VLPs). The *Helix pomatia* lectin, which is specific for terminal N-acetylgalactosamine residues in α linkages, and biotinylated *Maackia amurensis* agglutinin (MAA), which is specific for sialic acid residues in α 2,3 linkages, were obtained from Biovalley SA (Marne la Vallée, France) and Oxford Glycosystems (Abingdon, United Kingdom), respectively. The CSLEX-1 monoclonal antibody (MAb) was obtained from BD Pharmingen (San Diego, CA). Peroxidase-labeled avidin was purchased from Vector Labs (Burlingame, CA), and the neuraminidase from *Arthrobacter tumeifaciens* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Synthetic oligosaccharides as polyacrylamide conjugates (PAA) or coupled to human serum albumin (HSA) were obtained from N. Bovin (Moscow, Russia) and Iso-SepAB (Tullingen, Sweden), respectively. The neoglycoconjugates examined in the present study were: H type 1-PAA (Fuca2Gal β 3GlcNAc β -R), H type 2-PAA (Fuca2Gal β 4GlcNAc β -R), H type 3-PAA (Fuca2Gal β 3GalNAc α -R), Le^x-PAA (Fuca2Gal β 4[Fuca3]GlcNAc β -R), α Gal-PAA (Gal α 3Gal β 4GlcNAc β -R), A tri-PAA (GalNAc α 3[Fuca2]Gal β -R), Le^x-HSA (Gal β 4[Fuca3]GlcNAc β -R), sialyl-Le^x-HSA (NeuAc α 2,3Gal β 4[Fuca3]GlcNAc β 3Gal β 4Glc β -R), and sialyl-LNnT-HSA (NeuAc α 2,3Gal β 4GlcNAc β 3Gal β 4Glc β -R).

Constructs containing open reading frames 2 and 3 were used to produce recombinant VLPs for genogroup I.1 (Norwalk virus, strain 8FIIa, GenBank accession number M87661.1) and genogroup II.4 (Houston strain, GenBank accession number EU310927) as previously described (6). Mutants in the P2 subdomain of VP1 were generated by Ala point substitution and used to produce recombinant VLPs His-329 (H329A), Asn-331 (N331A), and Trp-375 (W375A) (11). After purification, the protein concentrations were determined, and the number of VLPs was calculated based upon the VP1 molecular weight and 180 copies of VP1 per virus particle (18, 39). Corresponding antibodies were produced by immunizing rabbits with the GI.1 or the GII.4 purified VLPs, respectively.

Oyster samples. A large batch of oysters (*Crassostrea gigas*) was purchased at the beginning of the study and placed in a clean area in Brittany. These oysters were used for ELISA, immunohistochemistry, and bioaccumulation experiments. All samples, each including at least eight oysters, were then randomly collected from this batch and shipped within 24 h to the laboratory at 4°C. Environmental data such as water temperature and salinity were monitored on a daily basis by using a Marel SMATCH TPS probe (NKE, Hennebont, France), located exactly on the same point as the oysters.

ELISA-based carbohydrate microtiter plate assays. Every month, eight oysters were shucked; digestive tissues (including the stomachs), gills, and mantles were dissected separately. One gram of each tissue was homogenized in 2 ml of phosphate-buffered saline (PBS; pH 7.4), heated for 10 min at 95°C, and centrifuged at 13,000 $\times$ g for 7 min at 4°C, and the supernatant was recovered. The protein concentration was estimated by using a BC Assay kit with bovine serum albumin (BSA) as a standard (Uptima, Montluçon, France). After adjustment at 40 μ g/ml, tissue extracts were coated onto Nunc Maxisorp immunoplates (ThermoFischer Scientific, Roskilde, Denmark) in 100-mmol/liter carbonate buffer (pH 9.6) by overnight incubation at 4°C. After blocking with 10% nonfat dried cow's milk in PBS for 1 h, VLPs were added at a final concentration of 0.2 μ g/ml (10⁷ particles per well), followed by incubation for 1 h at 37°C. The plates were then incubated with VLP-specific antibodies at a 1/1,000 dilution in PBS–5% milk for 1 h at 37°C, and then peroxidase anti-rabbit IgG (Uptima) at 1/2,000 in PBS–5% milk was added, followed by incubation for 1 h at 37°C. Between each step, plates were washed three times with PBS containing 5% Tween 20 (Sigma-Aldrich, France). The enzyme signals were detected with TMB (3,3',5,5'-tetramethylbenzidine; BD Bioscience, San Jose, CA) as a substrate and read at 450 nm with a spectrophotometer (Safire; Tecan).

Sodium periodate treatment was performed on the coated material using 10 mmol of sodium periodate/liter in a 50-mmol/liter sodium acetate buffer (pH 5.0) for 30 min at room temperature, followed by a 10-min incubation with 1% glycine in water. Control wells were treated similarly without sodium periodate. Inhibition of VLP binding with the *H. pomatia* lectin was performed by prein-

cubation with the lectin diluted to 50 μ g/ml in PBS for 30 min at room temperature. After several washings, VLPs were added, and their binding detected as described above.

For each tissue extract, negative controls without VLPs but with primary or secondary antibodies were included. Human secretor type A, B, and O saliva samples coated in individual wells on the ELISA plate served as positive controls in each plate. The GI.1 VLPs are known to bind well to A and O secretor-positive saliva but poorly to B secretor-positive saliva (31), and the GII.4 VLPs used here bind well to secretor-positive saliva irrespective of the ABO type (unpublished results). For the *H. pomatia* inhibition test, positive (without *H. pomatia* preincubation) and negative controls (without VLPs), as well as positive controls on saliva samples, were tested on the same plate. In all cases, after validation of the saliva controls and the negative controls, the optical density (OD) values were measured for all samples. All samples were analyzed in duplicate on the same plate, and discordant results between the two replicates (a >0.1 OD value difference) were not accepted. The test sample ratio was determined by dividing the OD value of the test sample by the OD value of the negative control (same tissue without VLPs). A sample was considered positive if this ratio was $\geq$ 2.

Binding of VLPs to immobilized neoglycoconjugates. Briefly, oligosaccharides conjugated to either PAA or HSA were coated onto Nunc Maxisorp immunoplates as previously described (31). After a blocking step with 5% defatted dry cow's milk, VLPs at 4 μ g/ml (2 $\times$ 10¹⁰ particles per well) were added for 2 h at 37°C. VLP binding was detected by incubation with the respective anti-GI.1 or anti-GII.4 rabbit antisera diluted at 1/1,000, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (Uptima). The peroxidase substrate TMB (BD Bioscience) was used, and the OD values were determined at 450 nm. OD values that were twice background were considered positive.

Bioaccumulation of VLPs. GI.1 wild-type and H329A, N331A, W375A mutant VLPs or GII.4 VLPs were added at various concentrations to 500 ml of clean seawater and then homogenized for 5 min. Four clean live oysters were added to the seawater, followed by incubation for 24 h at 15°C (water temperature) under oxygenation. All VLPs were bioaccumulated twice (GI.1 mutants), five times (GI.1 wild type), and seven times (GII.4), and the different bioaccumulations were conducted from October to April (except December).

Immunohistochemical analysis. Oysters (uncontaminated or after bioaccumulation) were shucked, and the body was cut horizontally so as to visualize all organs (from the mouth to the anus) on a single section. Sections were fixed in 10% formaldehyde (Gurr, VWR, France) for 24 h, paraffin embedded, and sliced into thin sections (5 μ m). After preparation of tissue sections as described previously (32), sections from uncontaminated oysters were covered with 2 μ g of GI.1 or GII.4 VLPs/ml (10¹¹ particles/ml) and left overnight at 4°C before being washed three times for 5 min each time in PBS at room temperature. The presence of VLPs bound to the oyster tissue was detected using the respective antibodies as previously described (32). Negative controls included sections from uncontaminated samples not exposed to VLPs and exposed sections without primary antibody. Staining with MAA was performed by incubating sections with the biotinylated lectin diluted at 10 μ g/ml overnight at 4°C, followed by a second incubation with peroxidase-labeled avidin at 1/1,000 for 45 min at room temperature. Immunoreactivity, detected under microscopic analysis, was considered strong (intense red coloration), weak (pale red coloration), or negative (no coloration).

For bioaccumulation experiments, bright-field images captured with an Olympus color charge-coupled device camera were analyzed by using a deconvolution algorithm to separate the dye contribution at each pixel (41). Regions of interest (covering hemocytes surfaces) were selected for each image through a markup algorithm in ImageJ.

Neuraminidase treatment was performed on oyster sections by incubation at 37°C with 15 mU of neuraminidase in 50-mmol/liter sodium acetate buffer (pH 5.3) for a total of 18 h with replacement of the neuraminidase solution every 6 h. Serial control sections were incubated in parallel in the same buffer without the enzyme. Blocking of VLP binding by the MAA lectin was performed by preincubating tissue sections with the lectin diluted at 50 μ g/ml in 1% PBS-BSA at 4°C overnight. Treatment with sodium periodate at 10 mmol/liter and 1 mmol/liter in 50 mmol/liter sodium acetate buffer (pH 5.0) was performed on tissue sections for 30 min at room temperature. Serial control sections were preincubated in the same conditions in the absence of the lectin or sodium periodate prior to addition of the VLPs. The sections were then rinsed three times with PBS and incubated with GI.1 or GII.4 VLPs for 1 h at room temperature, and the detection of binding was performed as described above.

Statistical analysis. Means were compared by using the Student *t* test, and a *P* value of <0.05 was considered significant (Origin Software, Paris, France).

TABLE 1. GI.1, GII.4, and mutant VLP binding to oyster tissue extracts evaluated by ELISA

VLP	No. of samples analyzed ^a	Mean S/N $\pm$ SD for various shellfish tissues ^b		
		Digestive tissues	Gills	Mantle
GI.1	50	7.4 $\pm$ 4.2	1.7 $\pm$ 0.5	1.8 $\pm$ 0.9
H329A	6*	0.8 $\pm$ 0.1‡	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
N331A	6*	10.1 $\pm$ 3.4†	2.4 $\pm$ 1.3	3.1 $\pm$ 1.8
W375A	6*	0.8 $\pm$ 0.1‡	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2
GI.4	50	10.8 $\pm$ 3.7	8.9 $\pm$ 4.3	9.9 $\pm$ 3.4

^a The mean OD values of saliva used as positive controls were 1.3 $\pm$ 0.3 for GI.1 and mutants and 1.0 $\pm$ 0.2 for GII.4. *, samples were selected from different periods of the year.

^b The mean signal-to-noise ratio (S/N), calculated as described in Materials and Methods, was obtained for all shellfish samples analyzed. †, comparison to GI.1, $P = 0.31$; ‡, comparison to GI.1, $P < 0.05$.

RESULTS

Comparison of capacities of genogroups to bind oyster tissue extracts. By using representative VLPs GI.1 and GII.4, the two main human NoV genogroups were compared in their capacity to bind to different oyster tissues, namely, the digestive tissues, the gills, and the mantle (Table 1). GI.1 VLPs bound readily to digestive tissues but not to the gills or the mantle. With GI.1 VLP, the mutants H329A and W375A lost their capacity to bind to digestive tissues, whereas the N331A mutant bound to digestive tissues. The ratio observed for this mutant was slightly higher than for the GI.1 prototype for all tissues tested, but the differences were not significant (Table

1). GII.4 VLPs bound strongly to digestive tissues but also to the gills and the mantle. Although the mean binding to the gills was somewhat lower than to the digestive tissues or the mantle, the difference did not reach statistical significance ($P > 0.3$).

In a previous study we demonstrated on tissues sections that GI.1 VLPs bind specifically to carbohydrate ligands of oysters digestive tissues (23). Since periodate oxidation cleaves C-C bonds with vicinal hydroxyl groups of carbohydrates, we treated the coated tissue extracts in the ELISA plate with 10 mmol of sodium periodate/liter and observed a total loss of binding capacity for GI.1 VLPs (Fig. 1A). Similarly, periodate treatment completely abolished the binding of GII.4 VLPs to all oyster tissue extracts, indicating that GII.4 binding was carbohydrate dependent, a finding similar to that seen with GI.1 (Fig. 1B).

Since the *H. pomatia* lectin, which recognizes α -linked *N*-acetylgalactosamine terminal residues, prevented binding of GI.1 to tissues sections (23), the ELISA plate was incubated with this lectin prior incubation with GI.1 or GII.4 VLPs. As expected, the GI.1 binding to digestive tissues was completely inhibited (Fig. 1C) ($P = 0.0124$). In contrast, the binding of GII.4 VLPs to oyster tissues (Fig. 1D) was only inhibited by ca. 50% for digestive tissues ($P = 0.0182$), and no significant inhibition was observed on the mantle and gills ($P = 0.453$ and $P = 0.315$, respectively). This suggested that GII.4 VLPs bind to two distinct ligands, one primarily or exclusively present in digestive tissues and shared with both those of *H. pomatia* and GI.1 VLPs and the other present in all three tissues but not shared with either *H. pomatia* or GI.1 VLPs.

FIG. 1. Inhibition of binding of VLPs to oyster tissue extracts. The binding of GI.1 (A and C) and GII.4 (B and D) VLPs to digestive tissues, gills, and mantle (x axis) tissue extracts pretreated with sodium periodate (A and B) or preincubated with the *H. pomatia* lectin (C and D) expressed as signal-to-noise ratios $\pm$ the standard deviation (SD) (y axis) was determined. Filled bars are homogenates in the absence of the inhibitory agent, and empty bars represent the presence of the inhibitory agents. The positive threshold is indicated by a dashed line.

FIG. 2. Binding of recombinant VLPs to neoglycoconjugates. The binding of GI.1 (■) and GII.4 (▒) VLPs to either PAA- or HSA-conjugated synthetic oligosaccharides (x axis) expressed as mean OD values of duplicates for each neoglycoconjugate (one representative experiment out of three with similar results) was determined.

Identification of the VLP ligands. In order to gain insight into the structure of the second carbohydrate ligand of GII.4 VLPs, we first tested the binding of the two strains to a selected set of neoglycoconjugates (Fig. 2). The GII.4 VLPs attached to H type 1, H type 3, and Le^x, as well as to the A epitope, a finding consistent with the recognition of a ligand shared with the *H. pomatia* lectin on oyster digestive tissues. In addition, and similar to what was previously observed with other GII strains (42), the GII.4 VLPs used in the present study attached to sialylated structures, sialyl-Le^x and, to a lesser extent, its nonfucosyl counterpart sialyl-LNnT (Fig. 2). These results suggested that the GII.4 VLPs may recognize a sialylated carbohydrate motif present in various oyster tissues.

To test this hypothesis, we used an immunohistochemistry approach. Oriented tissue sections with all organs present on the same slide were incubated with the different VLPs. The GI.1 VLPs bound principally to the esophagus, midgut, and primary and secondary ducts of the digestive diverticula and tubules, as previously described (23, 54) (Fig. 3A). No binding was observed to the gills, mantle, or labial palps (Fig. 3A and C). The GII.4 VLPs also bound to the different digestive organs, but strong binding was additionally observed to the labial palps, gills, and mantle (Fig. 3B, D, and E). In contrast to what was observed with GI.1 VLPs, those from the GII.4 strain bound to nonepithelial histological structures from all organs in addition to the digestive epithelial cells. This set of observations is in accordance with the results obtained by ELISA and shown in Table 1.

After treatment of tissue sections with 1 mmol of sodium periodate/liter, GI.1 binding was not clearly decreased (data not shown), and some binding was still observed on the epithelial part of the digestive tissues for GII.4, although it was no longer detectable on gills or other tissues (Fig. 3F and G). However, when tissue sections were treated with a higher periodate concentration (10 mmol/liter), both GI.1 (data not shown) and GII.4 binding were completely lost (Fig. 3H). This result is consistent with the possible presence of two GII.4 ligands on oyster tissues, one localized in all tissues and sensitive to periodate at 1 mmol/liter, as expected for a sialic acid-containing epitope, and the second restricted to the digestive tissues and only sensitive to a higher periodate concentration, as expected for a neutral carbohydrate epitope. To

determine whether sialic acid residues in α 2,3 linkage were indeed present in *Crassostrea gigas* oysters, tissue sections were first incubated with the MAA lectin and an anti-sialyl-Le^x MAb. Although no reactivity was detected using the anti-sialyl-Le^x, a strong staining, largely overlapping with that of the GII.4 VLPs, was observed in most tissues with MAA (Fig. 3I). In addition, after treatment of the tissue sections with neuraminidase, MAA binding was completely lost, except on the mantle single epithelial cell layer, indicating that on most histological structures, the lectin recognized neuraminidase-sensitive sialylated structures, as expected (Fig. 3J). When tissue sections were incubated with the MAA lectin prior to GII.4 VLP incubation, the VLP binding was drastically reduced, showing a competition between the lectin and the VLPs for their binding sites (Fig. 3K and L). To confirm the involvement of sialic acid residues in GII.4 VLPs binding, tissue sections were then treated with neuraminidase before incubation with VLPs. The attachment of GII.4 VLPs was largely decreased (Fig. 3M and N). In contrast, no effect was observed on GI.1 VLP binding (Fig. 3O).

Bioaccumulation. In order to evaluate biological impact of live oysters, bioaccumulations were performed with the different GI.1 VLPs, using final concentrations ranging from 5×10^5 to 5×10^8 VLPs/ml of seawater. GI.1 VLPs were bioaccumulated very efficiently and were detected only in digestive and connective tissues. An intense and clear red staining of digestive tubules (Fig. 4F) or some cells within connective tissues (Fig. 4D) was observed. Even at 10^5 VLPs/ml of seawater, VLPs could be detected after thin-layer sectioning. Surprisingly, GII.4 VLPs were not found in oyster tissues after bioaccumulation, despite several assays and high concentrations (10^9 VLPs/ml of seawater) used. Subsequent studies demonstrated that the GII.4 VLPs lost their structural integrity when suspended in seawater (electron microscopy observation [data not shown]). Since differences were observed between GI.1 and mutants by ELISA, bioaccumulations were conducted with the three GI.1 mutant VLPs. All three mutants could be detected within shellfish tissues, but only when higher concentrations were used (at least 10^9 VLPs/ml of seawater). Specific staining was seen mainly on hemocytes localized in the connective tissue of all organs. No red or brown color was seen in the negative controls (Fig. 4A and B). Nevertheless, different color intensities were clear under microscopic examination after bioaccumulation of either the wild type and N331A mutant or the H329A and W375A mutants: the H329A and W375A mutants showed pale brown coloration (Fig. 4C and E), whereas the wild type and N331A mutant showed a clear red coloration (Fig. 4D and F), suggesting a higher bioaccumulation efficiency of the latter. The dye color intensity was computed for 307 hemocytes from 9 individual fields from tissue sections of oysters bioaccumulated with GI.1 wild-type VLPs at 10^7 VLPs/ml, for 826 hemocytes from 13 fields from tissue sections of oysters bioaccumulated with the GI.1 H329A mutant VLPs at 10^9 VLPs/ml, and for 148 hemocytes from 6 fields of control oysters. The mean intensities showed a statistically significant difference ($P < 0.05$) between the GI.1 wild-type and H329 VLPs (Fig. 5). Although dye intensity cannot be directly correlated with the number of bioaccumulated VLPs, this result clearly shows that the lack of ligand recognition drastically reduces bioaccumulation. This is particularly obvi-

FIG. 3. Immunohistochemical analysis of GI.1 and GII.4 recombinant VLP binding to oyster tissues. VLPs and the MAA lectins were incubated on tissue sections as described in Materials and Methods. (A) Binding of GI.1 VLPs to the esophagus (arrow) and lack of binding to the labial palps (star). (B) Binding of GII.4 VLPs to the esophagus (arrow), the labial palps (star), and connective tissue structures (arrowhead). (C) Absence of binding of GI.1 VLPs to the gills. (D) Binding of GII.4 VLPs to connective tissue structures of the gills (arrowhead) and weak staining of epithelial cells (arrow). (E) Binding of GII.4 VLPs to the mantle. (F to H) Staining of the digestive tissue by GII.4 VLPs on epithelial cells (arrow) and connective tissue (arrowhead) in the absence of periodate treatment (F), after 1 mM periodate treatment (G), and after 10 mM periodate treatment (H). (I and J) Staining of the digestive tissue by MAA without (I) and with (J) neuraminidase treatment. (K and L) GII.4 VLP binding to the gills without (K) or with MAA preincubation (L). (M and N) GII.4 VLP binding to the digestive tube without (M) and with (N) neuraminidase treatment. (O) GI.1 binding to the esophagus after neuraminidase treatment.

FIG. 4. Immunohistochemical analysis of GI.1 and GI mutants recombinant VLPs after bioaccumulation. Oysters were incubated in seawater seeded with GI.1, or H329A, for 24 h. The tissues were then fixed, and VLPs were detected by immunohistochemistry as described in Materials and Methods. Negative controls were performed with tissue sections from oysters bioaccumulated with GI.1 VLPs but without primary antibody (A) (an arrow indicates hemocytes that appear after counterstaining with hemalun) or using tissue sections from nonbioaccumulated oysters incubated with both primary and secondary antibodies (B). Wild-type GI.1 VLPs after bioaccumulation were detected in connective tissues (D) and around digestive diverticula (F); H329 VLPs were detected in connective tissues (C) and around digestive diverticula (E). Note the difference in red color intensity between panels D and F and between panels C and E.

FIG. 5. Quantitative analysis of immunohistochemical detection of bioaccumulated VLPs. Dye color intensities of hemocytes were computed as described in Materials and Methods. Mean values $\pm$ the SD are shown for hemocytes of oysters bioaccumulated with wild-type Norwalk virus VLPs (NVwt) at 10^7 VLPs/ml, with mutant H329A Norwalk virus VLPs (H329) at 10^9 VLPs/ml, or of a control oyster (control). *, $P < 0.01$; **, $P < 0.001$.

ous when considering that mutant VLPs were detected only when seeded at a concentration a hundred times higher than that of the GI.1 prototype VLPs.

Seasonal variations. Oysters collected every month were assayed by ELISA at least three times each and in separate experiments. A clear seasonal effect was observed for GI.1 VLPs, with an increased binding capacity during the months from January to May that correspond to the end of winter and most of spring (Fig. 6A). A comparison of the two defined periods, period A (January to May) (mean signal-to-noise ratio, 10.9 ± 3.6) versus period B (June to December) (4.4 ± 1.1), revealed a highly significant different level of GI.1 binding to oyster digestive tissues ($P = 4.8 \times 10^{-12}$). In contrast, for GII.4 VLPs, seasonal variation of binding to tissue extracts was far less apparent (Fig. 6B), although the differences between these two periods (12.3 ± 3.7 compared to 9.1 ± 2.8), were statistically significant in the digestive tissues ($P = 0.001$), mantle ($P = 0.004$), and gills ($P = 0.01$). No significant differences were observed between the binding of GI.1 and GII.4

FIG. 6. Seasonal variations of VLP binding to oyster tissue extracts. The binding of VLPs from the GI.1 strain (A) and the GII.4 strain (B) to digestive tract (■), gill (▒), and mantle (□) extracts from collected oysters over 1 year (x axis), expressed as the signal-to-noise ratio $\pm$ the SD (y axis), was determined. The positive threshold is indicated by a dashed line.

VLPs to the digestive tissues during period A ($P = 0.167$), but a significant difference was observed for binding to the digestive tissues during period B ($P = 1.28 \times 10^{-10}$). The seawater temperature varied from 6 to 20°C and showed statistically significant different values between the two seasons considered (11.56 ± 2.8 compared to 16.8 ± 2.9 , $P < 0.001$), suggesting a potential inverse association with high expression of GI.1 binding sites on the oyster gut. However, the water temperature began to decrease about 2 months prior to the appearance of strong GI.1 binding, showing a loose association (Fig. 7). Salinity presented a more complex pattern of variation, with its highest level being in the summer. Although it was also lowest during cold months (December and January), presumably in association with other environmental parameters such as rain, this was not clearly associated with virus binding.

DISCUSSION

Noroviruses are genetically and antigenically diverse (3, 15). Since the first characterization of the Norwalk virus in 1990 (17), a large number of strains have been described worldwide. The genetic classification system is based on the relatedness of the complete VP1 capsid protein, and currently there are five recognized genogroups (58). Among these five genogroups,

humans may be infected by GI, GII, and GIV strains, whereas GIII and GV strains infect cows and mice, respectively. For some years the GII strains, particularly those of the GII.4 cluster, have been the predominant viruses detected in different parts of the world (29, 46). In addition to this high prevalence of GII.4 NoV in the human population, another important distinctive feature of GII.4 is their manner of transmission

FIG. 7. Salinity and temperature variations. Salinity (□) and temperature (▲) were expressed as mean values of daily recorded data $\pm$ the SD per month.

since they appear to be mainly transmitted via person-to-person contact in community outbreaks (47). Other strains, and importantly GI strains, are more often transmitted via food or environmental contamination (30, 36). Fecal viral load (which is higher for GII strains than for GI strains) (10), shedding by asymptomatic subjects (2), and distinct behaviors during wastewater treatments (12) or food processing (8) may partially explain some of the observed differences between the GI and GII epidemiologies. More general parameters, such as global climate change, may also have an impact on outbreak seasonality and strain transmission (40), and the overall impact of environment on infectious diseases needs to be considered (43). However, the epidemiological difference between genogroups implicated in outbreaks is even clearer when considering oyster-related outbreaks where NoV GI may constitute up to 30% of strains detected in patient stools or shellfish samples (14, 19, 21, 24, 35). For a long time, oysters were believed to act as filters or ionic traps, passively concentrating particles. However, depuration failure, long-term persistence in shellfish, and the above-mentioned difference in strain transmission argue in favor of more specific mechanisms for NoV bioaccumulation in oysters. Specific binding of Norwalk virus via a carbohydrate structure very similar to the human histo-blood group A antigen in *Crassostrea gigas* oysters (23), subsequently confirmed to occur in another oyster species (*Crassostrea virginica*) (52), may explain these differences. Interestingly, characterization of a blood group A activity in the acidic polysaccharide fraction from *Crassostrea gigas* viscera was reported quite a long time ago (38). The data presented here confirmed that GI.1 VLPs bind mainly to digestive tissues but not to other organs and were consistent with the results of bioaccumulation studies, performed with Norwalk virus (GI.1) and reverse transcription-PCR detection (4). However, genetic diversity of NoVs is also reflected in their binding capacity to various HBGA structures (26, 54). Differences observed between GI.1 and GII.4 binding to human HBGA are also present in oyster tissues. Our data demonstrate that the distribution of GII.4 is not restricted to digestive tissues as for GI.1, in accordance with reports demonstrating the presence of GII.4 in gills, albeit to a lower extent than in digestive tissues (33, 56). Our quantitative analysis is consistent with a lower expression of GII.4 binding sites in gills compared to the digestive tissues, although the difference was not statistically significant. In addition, we demonstrate here that the binding to gills and mantle tissue sections involves a sialic acid in $\alpha 2,3$ linkage, whereas in digestive tissues the interaction involves both the sialic acid and an A-like carbohydrate ligand. Very little is known regarding sialic acid and its distribution in oyster tissues (55). Some differential recognition of these ligands by GI and GII strains may lead to distinct outcomes in terms of the persistence of viral particles within the different organs. In other words, recognition of the sialylated ligand by GII strains may lead to a quicker degradation or release, whereas recognition of the A-like ligand results in virus persistence. After accidental contamination by sewage of a producing area a few years ago, we found that after 1 week the number of shellfish containing GII NoVs was higher than for GI strains, whereas after 3 weeks the converse was true (24). Although this hypothesis remains somewhat speculative and requires further evaluation, it may partly explain why GII strains that are shed in the environment

in far larger amounts than GI strains are relatively less common causes of oyster-related outbreaks (14, 19, 21, 24, 35).

In addition to ligand-specific recognition, binding of NoVs to oyster tissues may involve nonspecific interactions, as suggested in the past for enterovirus or reovirus (5, 7, 13). However, it is likely that these nonspecific mechanisms of attachment are less efficient than specific ligand-mediated binding, and we may predict that viruses captured through nonspecific interactions only will be less efficiently concentrated and/or that their persistence within oyster bodies will be shorter. The demonstration that GI.1 mutant VLPs were accumulated confirmed this hypothesis since the two mutants that did not recognize the type A antigen were detected in tissue sections, albeit to a far less extent than the GI.1 controls VLPs. These mutants were made because the individual amino acids make up (H329 and W375) or are in proximity to (N331) the site of the VLP responsible for binding to HBGA for Norwalk virus, based upon studies performed at an atomic resolution scale. Mutation of His-329 or Trp-375 chains completely abrogates carbohydrate binding (11). Similarly, these mutations induced a total lack of recognition of oyster digestive tissues and a less efficient bioaccumulation. Another consequence of specific ligand-mediated bioaccumulation may be its effect on viral persistence within the shellfish body. This may be the objective of future studies, since VLPs, being noninfectious, can be used to bioaccumulate oysters and then relocated in the environment for a follow-up analysis (28). Unfortunately, the GII.4 VLPs used in the present study were not stable after dilution into seawater (data not shown), so bioaccumulation could not be performed. Although VLPs are useful surrogates of the non-cultivable NoVs, control experiments must be performed before use of the VLPs in environmental studies to avoid misinterpretation of negative results. The bioaccumulation experiments showed that VLPs are localized in hemocytes within connective tissues. Two subtypes of these cells have been defined, the granulocytes and the hemoblastlike cells. Granulocytes have important physiological functions, including nutrient transport, digestion, wound healing, shell mineralization, and excretion, suggesting that viral particles could be destroyed rapidly if ingested by this cell type (1). However, hemoblastlike cells do not contribute to defensive responses such as phagocytosis or encapsulation and are lacking common intracellular enzyme systems associated with host defense (1). Further characterization of these cells will be important for evaluating viral particle behavior.

Another major interest of the data presented here concerns the observed seasonal variation. Oysters are able to bind much more efficiently to GI.1 VLPs during the first 5 months of the year (January to May) compared to the rest of the year. At variance with our results, a lack of seasonal variation was reported for three oyster species using GI.1 VLPs (53). Different parameters such as the number of replicates, the sensitivity of the test, or environmental conditions may explain the different results. The differences observed here may be linked to the water temperature, which is lower during the end of winter and beginning of spring. The influence of water temperature is difficult to analyze since many other environmental parameters, such as oyster physiology, the chlorophyll *a* concentration, and the presence of phytoplankton, may also have an impact (16). Interestingly, the end of winter/beginning of

spring period corresponds to the highest concentration of NoVs in sewage and to a time when heavy rainfall is common, both of which may increase the risk of oyster contamination following the failure of a sewage treatment plant or during flooding (12, 37). Although present, the seasonal effect was markedly less apparent for GII.4 VLPs, suggesting that the corresponding strains may be accumulated with more or less similar efficiency all year round. In France, the peak of oyster-related outbreaks occurs at the beginning of the year (21, 22, 24, 25), whereas the peak of consumption lies between December 24 and 31 (45).

We demonstrated here that NoVs of distinct genogroups present a different behavior with regard to oyster tissue recognition, a behavior similar to findings in human tissues, and that seasonal variation of NoV VLP binding to oyster tissues exists. As fundamental studies progress to understand the behavior of NoVs in humans, basic research on oyster contamination mechanisms will progress as well. Understanding the processes and mechanisms of virus uptake by molluscan shellfish, in conjunction with environmental studies, will lead to the development of strategies to prevent oyster contamination in the future. These studies, initialized more than 40 years ago for other enteric viruses, may be more successful now as new tools are developed.

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