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Precise Characterization of Norovirus (Norwalk-Like Virus)-Specific Monoclonal Antibodies with Broad Reactivity

Tomoko Yoda,[1]* Yasuhiko Suzuki,[2]† Yoshitake Terano,[3] Kenji Yamazaki,[4] Naomi Sakon,[4] Tsuyoshi Kuzuguchi,[5] Hajime Oda,[6] and Teizo Tsukamoto[1]

Division of Food Microbiology,[1] Division of Pathology,[2] Division of Virology,[4] and Division of Industrial Health,[6] Osaka Prefectural Institute of Public Health, and Department of Pharmacology, Osaka University of Pharmaceutical Sciences,[3] Osaka, and Department of Molecular Microbiology, Gifu Prefectural Institute for Bio-Industrial Technology, Gifu,[5] Japan

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We have been characterizing monoclonal antibodies against Norovirus (Norwalk-like virus). In the course of our study, two monoclonal antibodies generated against Norovirus genogroup II capsid protein were found to react not only to genogroup II but also to genogroup I recombinant capsid proteins. In addition, we showed that these two monoclonal antibodies reacted to a 40-amino-acid-fragment located close to the N-terminal region of genogroup II Norovirus. Similar reactivity was observed with the equivalent region of genogroup I Norovirus. In this study, we confirmed that the epitopes of the two monoclonal antibodies existed within an 11-amino-acid peptide. To obtain an idea of the reactive ranges of the two monoclonal antibodies toward different strains of Norovirus, their reactivities were investigated using 16 types of peptide constructed according to the data in GenBank and 8 recombinant capsid proteins (7 whole capsid proteins and 1 short [80-amino-acid] protein fragment). A characteristic broad reactivity of the two monoclonal antibodies is clearly shown by the results of this study. Thus, these monoclonal antibodies could be useful tools for detecting a broad range of Norovirus strains.

Norwalk virus (NV) is a member of the family *Caliciviridae*, genus *Norovirus*, formerly called *Norwalk-like virus* and possessing a single-stranded RNA genome. NV has been a significant cause of nonbacterial infectious gastroenteritis and food-borne diseases all over the world. The virus is highly infectious and spreads through contaminated food as well as water in food-borne disease cases. In infectious gastroenteritis cases, this virus is spread from person to person within semiclosed communities such as schools, nursing homes, and hospitals. According to national food-borne disease statistics in Japan, the number of patients involved in incidents caused by NV is likely to be large. Thus, diagnosis of NV infection is extremely important for public health, since strategies for treatment of patients and control of diseases will be carried out effectively when the causative agent is diagnosed. NV has been diagnosed using electron microscopy (EM), reverse transcription-PCR (RT-PCR), and enzyme-linked immunosorbent assay (ELISA). These methods have worked efficiently in most cases. However, due to the great diversity of nucleotide sequences throughout the whole genome of NV and the capsid protein, neither ELISA nor RT-PCR detects all types of NV (3, 4, 5, 9). In addition, the very limited numbers of particles shed in patient fecal material makes detection by EM difficult (2, 9). In cases of NV infections with homologous strains, genomic RNA detection by RT-PCR and antigen-antibody detection by ELISA can yield excellent results (1, 3, 4, 7, 8).

Regardless of its variation, NV has been divided into two large genogroups, genogroup I (GI) and genogroup II (GII). We expressed a large amount of several strains of NV capsid protein in an *Escherichia coli* system and generated monoclonal antibodies (MAbs) against GI capsid protein (12) as well as GII capsid protein (11). Two MAbs generated against recombinant GII capsid protein (recombinant NV36 [rNV36] capsid protein) reacted to recombinant capsid proteins of both genogroups as shown in the previous study of Yoda et al. (12). In the present study, we demonstrated the broad reactivity of the two MAbs by using 24 different types of NV stretches or whole recombinant capsid proteins corresponding to the epitope regions of these two MAbs based on the data available in GenBank and expressed in an *E. coli* system.

MATERIALS AND METHODS

Virus, plasmid, and *E. coli*. NV strains 36, 21, 114, 96-908, 99-8, 002, and Gifu'96 were isolated, and recombinant capsid proteins of these strains were prepared as described previously (11, 12). pT7 Blue was used as a TA cloning vector (Takara Biomedicals, Kusatsu, Japan). The pTrx-Fus expression vector and anti-NV36 MAbs used in this study were also prepared as described previously (11, 12). Another NV strain, 00-013, was isolated from a patient in our institute. The pTrx-FusH expression vector, a derivative of pTrx Fus (Invitrogen, Carlsbad, Calif.) carrying a nucleotide sequence encoding a hexahistidine tag in a multicloning site (between *Sal*I and *Pst*I), was used in the present study. *E. coli* strains GI724 and XL1-Blue were used as host cells for pTrx-Fus, pTrx-FusH, and pT7 Blue.

Construction of the NV fragments in pTrx-FusH expression vectors. The pTrx-FusH expression vector was used to construct fusion proteins with thioredoxin (TRX) at the N-terminal region and a hexahistidine tag at the C-terminal region. Fragments of different NV strains were expressed between TRX and the hexahistidine tag. The oligonucleotides used in this experiment were designed as follows. All oligonucleotides had sticky *Bam*HI sites at the upstream end and sticky *Sal*I or *Xba*I sites at the downstream end. Each pair of oligonucleotides was designed on the basis of the objective peptide (amino acid sequence) shown in Fig. 2c. The oligonucleotide mixture consisted of 20 μl each of the forward and

* Corresponding author. Present address: Division of Bacteriology, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinariku, Osaka City 537-0025, Japan. Phone: 81-6-6972-1321, ext. 361. Fax: 81-6-6972-2393. E-mail: yoda@iph.pref.osaka.jp.

† Present address: Department of Microbiology and Pathology, Faculty of Medicine, Tottori University, Yonago City, Japan.

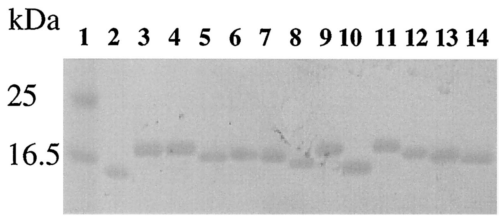


FIG. 1. Purification of recombinant proteins. Fusion proteins were purified from *E. coli* lysates by using Ni-agarose or Q-Sepharose column chromatography. The purity of each stretch was analyzed by SDS-PAGE. Lane 1, marker; lane 2, TRX (1 μg); lanes 3 to 14, recombinant fusion stretches S9 (1 μg), S10 (1 μg), S11 (0.5 μg), S12 (0.8 μg), S13 (0.8 μg), S14 (0.6 μg), S15 (1 μg), S16 (1 μg), S17 (1 μg), A1 (0.8 μg), A2 (0.8 μg), and A3 (1 μg), respectively.

reverse nucleotide solutions (10 μM); it was incubated at 72°C for 5 min and cooled down slowly to room temperature for annealing. The annealed oligonucleotides were then ligated with *Bam*HI- and *Sal*I- or *Xba*I-digested pTrx-FusH vectors. The ligation mixtures were used to transform GI724 competent cells. For the rNV strain 00-013, a different cloning strategy was used. PCR consisted of 30 cycles of denaturation (94°C for 30 s), primer annealing (55°C for 30 s), and extension (72°C for 60 s), using primers SRSVI-L1 *Bam*HI (5′-CGGGATCCA TGATGATGGCGTCTAAGGAC-3′) and GI F2R *Sal*I (5′-ACGCGTCGACA TCACCGGGTGTATTGTTAGG-3′) with the template (a PCR product amplified with primers G1F1 and G1R1 [6]). The PCR products of 00-013 were inserted into pT7 Blue, followed by sequence confirmation as described previously (10). The insert of 00-013 was cut out from the plasmid with *Bam*HI and *Sal*I and was ligated with pTrx-FusH as described above.

Induction and analysis of recombinants. The resulting colonies carrying the expression constructs were incubated, induced, and analyzed as described previously (10). Expression products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Both strands of the insert and junction of recombinant plasmids were sequenced by using the PRISM Dye-Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif.) with Trx-up and Trx-dw and were analyzed with the model 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems) as described previously (11).

Production and purification of recombinant proteins. Recombinant peptides were produced from a 50-ml tryptophan-induced culture of transformed *E. coli* as described previously (10). Cells were harvested and suspended in 2 ml of a native-condition lysis buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 5 mM imidazole). After sonication, lysates were centrifuged at 9,000 × *g* for 15 min. The supernatant was incubated with nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) for 30 min at 4°C. The nickel-agarose was packed into a column and washed with the native-condition lysis buffer. Histidine-tagged peptides were eluted with an elution buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 60 mM imidazole). The purity of the recombinant peptides was examined by SDS-PAGE.

S13 was purified by using a Q-Sepharose column, because this recombinant peptide was not bound to the nickel-agarose column. After sonication, the lysate was diluted 10 times with distilled water, and then the buffer was adjusted to 20 mM Tris-HCl (pH 7.6) by addition of 1 M Tris-HCl buffer for Q-Sepharose column chromatography. After the lysate of S13 was bound on the Q-Sepharose column, the column was washed with washing buffers A (20 mM Tris-HCl [pH 7.6]) and B (20 mM Tris-HCl [pH 7.6]–100 mM NaCl). The recombinant peptide S13 was eluted with an elution buffer (20 mM Tris-HCl [pH 7.6]–200 mM NaCl).

ELISA. A direct ELISA was performed to determine the reactivity of the two broadly reactive MAbs. Briefly, 96-well microplates were coated with 1 μg of an antigen and incubated overnight at 4°C in carbonate buffer (10). After four washes with 0.05% Tween 20 in Tris-buffered saline (TTBS), microplates were incubated with the MAbs at various concentrations (10 ng/ml for 1B4; 20 ng/ml for 1F6) for 1 h at 37°C. After a wash with TTBS, horseradish peroxidase-labeled anti-mouse immunoglobulin G (Fab specific; product A2304, lot 121K4844; Sigma, St. Louis, Mo.) diluted at 1:5,000 was used as the secondary antibody. After incubation for 1 h at 37°C, bound antibodies were detected as the activity of horseradish peroxidase by addition of 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate, followed by incubation for 30 min at room temperature. The enzyme reaction was then stopped with 1 M phosphoric acid, and absorbance was measured at 450 nm in a model 550 microplate reader (Bio-Rad, Richmond, Calif.). All reactions in ELISAs were carried out in triplicate.

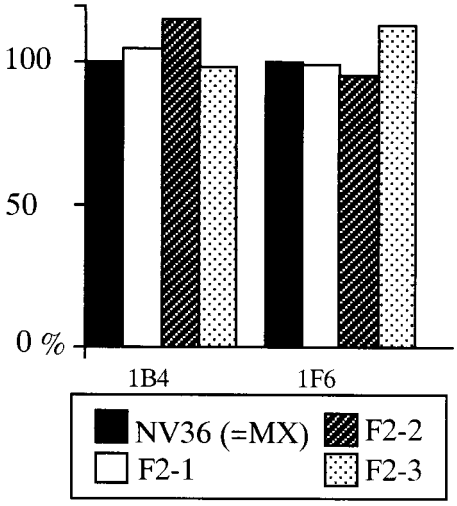
RESULTS

Recombinant peptides. Twenty-three pTrx-FusH recombinant plasmids were constructed by insertion of oligonucleotide pairs encoding 21, 11, or 5 amino acids, and fusion peptides were produced as described in Materials and Methods. Some of the purified fragments are shown in Fig. 1. SDS-PAGE analysis was performed to estimate the concentrations of purified fragments by visual comparison with known quantities of TRX. Amounts of recombinant fragments obtained from 50-ml cultures ranged from 0.2 to 7.5 mg.

Characterization of fine epitopes of broadly reactive NV-specific MAbs. To examine fine epitopes of the two MAbs, their reactivities to three types of 21-amino-acid fragments overlapping each other were measured by ELISA. The two MAbs recognized all of the fragments similarly, as shown in Fig. 2a. The common sequence stretches in these three 21-amino-acid fragments consisted of 11 amino acids, as shown in Fig. 2b. The 11-amino-acid stretch termed S1 is considered to have an epitope(s) of the two MAbs, and their reactivities to S1 by ELISA confirmed this, as shown in Fig. 2c. Since the reactivities of the two MAbs toward S1 and NV36 (recombinant whole capsid protein, MX type) appeared to be similar, we decided to compare the reactivities at the molecular level.

FIG. 2. (a) Reactivities of MAbs toward three 21-amino-acid stretches. The reactivities of MAbs 1B4 and 1F6 against fragments F2-1, F2-2, and F2-3 were measured by ELISA and are expressed as percentages. The optical density (OD) of TRX was subtracted from that of each sample. The OD of rNV36 was considered to be 100%, and the percentage of reactivity was calculated as (OD of sample) × 100/(OD of rNV36). (b) Relationship of the 40-amino-acid fragment 2 with the three 21-amino-acid stretches used in the ELISA for which results are shown in panel a. The underlined amino acid sequences are the unique part of fragment 2 (other parts overlap with proximal fragments). (c) Comparison of the reactivities of MAbs 1B4 and 1F6 toward various rNV capsid proteins. Under "Category" on the left, "Epitope" indicates that the 11-amino-acid stretch S1, which is part of the rNV36 whole capsid protein, is the epitope of the two MAbs. NV36 (MX) had been used for generating these two MAbs, so it was used as a positive control in this experiment. Modified stretches (S2 to S6) were constructed by slight modifications from the original data available in GenBank. GI proteins (S7 to S12) were constructed based on GenBank data for GI type NV strains. Recombinant fragment 00-013 (80 amino acid residues) was constructed from the NV clinical isolate 00-013. GII proteins (S13 to S17) were constructed based on GenBank data for GII type NV strains. A1 is a 5-amino-acid stretch that is conserved among NV strains. Artificial stretches (A2 and A3) are artificially constructed fragments that are supposed to have a conformation completely different from those of clinical NV strains. Representative strain names, where available, are given, as are amino acid sequences of the positions equivalent to those of S1 in each peptide. The reactivities of 1B4 and 1F6 were examined by ELISA, and the strength of each reaction was expressed as a percentage as described for panel a. Accession numbers of the NV strains listed in this figure are as follows: for KU8GI, AB067547; for Desert Shield (DS), U04469; for Stav/95/Nor, AF145709; for BS5, AF093797; for Southampton (SOU), L07418; for KU24aGI, AB067549; for KU4bGI, AB058536; for Camberwell, AF145896; for Hawaii, U07611; for KU17GII, AB058556; for KU44GII, AB058581; and for Alphatoron98, AF195847.

(a)

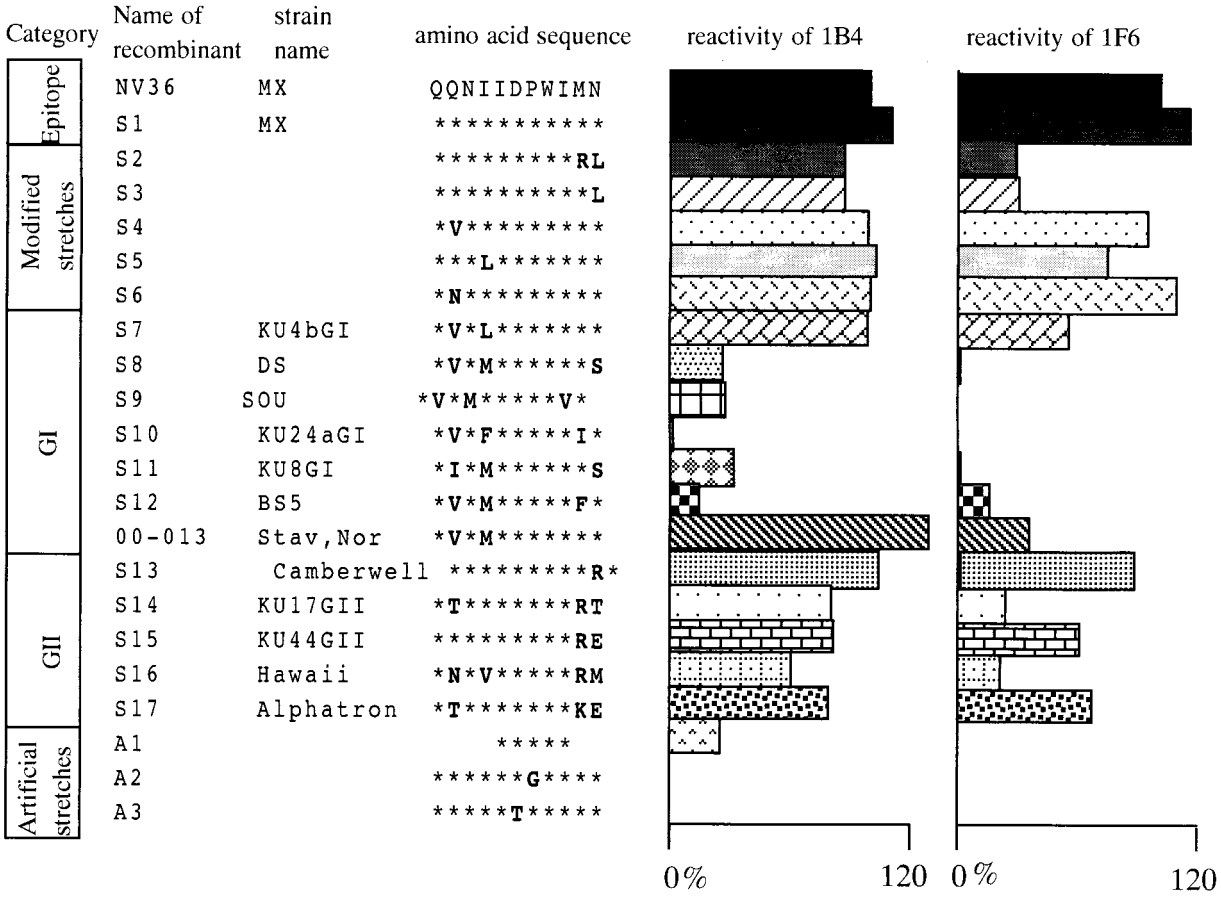


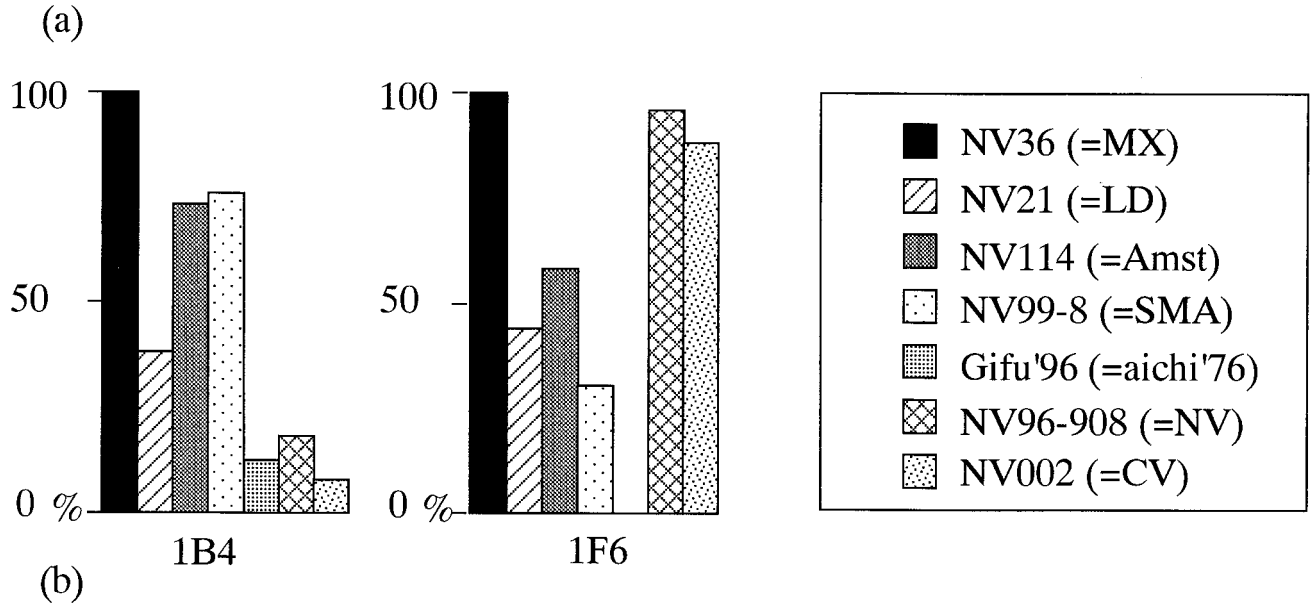
(b)

fragment 2: PVAGAAIA<u>APLTGQQNIIDPWIMNNFVQA</u>PGGEFTVSPRN

F2-1: APLTGQQNIIDPWIMNNFVQA
F2-2: GAAIAAPLTGQQNIIDPWIMN
F2-3: QQNIIDPWIMNNFVQAPGGEF

(c)





(b)

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NV36 (MX type):                PVAGAAIA APLTGQQNIIDPWIMNNFVQA PGGEFTVSPRN
NV21 (LD type):                **V***** **VA****V*****R****** **********
NV114 (Amsterdam type):        *****SL* **VV**L*******R****** *A*********
NV99-8 (SMA type):             ******L* **V***T*******RA***** *N*********
Gifu'96 (Aichi'76 type):*****S** *****N*******RL***** *N*********
NV96-908(NV68 type):           ****SST* VATA**V*P*****I****** *Q****I**N*
NV00-002(Chiba type) :         ****SST* LATA**V*L*****I****** *Q****I**N*
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FIG. 3. (a) Comparison of the reactivities of MAbs 1B4 and 1F6 toward various rNV capsid proteins. Reactivities were measured by ELISA and are expressed as percentages. The optical density (OD) of TRX was subtracted from that of each sample. The OD of rNV36 was considered to be 100%, and the percentage of reactivity was calculated as (OD of sample) × 100/(OD of rNV36). Strain types MX, LD, Amsterdam, SMA, and Aichi'76 are categorized as GII, while types NV68 and CV are categorized as GI. (b) Sequence alignment of the various rNV capsid proteins used in the ELISA for which results are shown in panel a. Amino acid residues corresponding to fragment 2 are given at the top. The epitope of the two MAbs (QQNIIDPWIMN; boxed) is within fragment 2 in NV36. The amino acid residues located at both edges of the box are considered to be recognition stretches of these two MAbs. Accession numbers of strains in this figure are as follows: Mexico (MX), U22498; Lorsdale (LD), X86557; Amsterdam98, AF195848; SMA, U70059; Gifu'96, AB045603; NV68, NC001959; Chiba (CV), AB022679.

The reactive patterns of these two MAbs were measured by ELISAs using either rNV fragment stretches or whole rNV capsid proteins and are shown in Fig. 2c and 3. That is, 16 different 11-amino-acid stretches (*E. coli*-expressed peptides corresponding to the S1 epitope) constructed based on the data available in GenBank were used as rNV stretch antigens (S2 to S17). Recombinants constructed from isolated strains, an 80-amino-acid fragment of 00-013 (Fig. 2c), and seven types of whole recombinant capsid proteins previously constructed (10) (Fig. 3) were used as antigens.

One of the MAbs, designated 1B4, bound to a conserved 5-amino-acid stretch (IDPWI, designated A1) but did not react to two artificially constructed stretches (A2 and A3) that had amino acid substitutions in the conserved region (Fig. 2c). 1B4 reacted to 23 different types of NV peptides and failed to react to 1 type of NV peptide, S11 (Fig. 2c). The other MAb, 1F6, failed to bind to the conserved 5-amino-acid stretch A1, to one rNV whole capsid protein (Gifu'96), and to four different stretches (S9, S10, S11, and S12) (Fig. 2c and 3).

DISCUSSION

We have been characterizing specific MAbs against NV in the expectation of finding broadly reactive MAbs that can eventually be used in diagnostic assays. The two broadly reactive MAbs 1B4 and 1F6 were generated against *E. coli*-expressed rNV36 whole capsid protein (GII, MX type) and reacted to fragment 2 (40 amino acid residues) in a previous study (11). To characterize fine epitopes of the two MAbs, three types of 21 amino-acid-stretches were constructed; from the results shown in Fig. 2a and b, an 11-amino-acid stretch (S1) was expected to carry their epitopes. The reactivities of the MAbs to S1 and to rNV36 whole capsid protein (Fig. 2c) confirmed the existence of their epitopes in the 11-amino-acid-

stretch S1. Even though these two MAbs reacted to the same region, they showed different reactivities toward 24 types of rNV capsid protein, including rNV stretches (17 types) (Fig. 2c) and rNV whole capsid proteins (7 types) (Fig. 3).

From its reactivities to the different fragments, MAb 1B4 is considered to recognize mainly the core part of the 11-amino-acid stretch, because it reacted to a conserved 5-amino-acid stretch (IDPWI) termed A1 but did not react to A2 and A3 (each of which has a single amino acid substitution in the 5-amino-acid stretch) (Fig. 2c). However, the reactivity of 1B4 appeared to be affected by changing other amino acid residues near this 5-amino-acid stretch, as with S11 (which has three amino acid substitutions near the conserved 5-amino-acid sequence).

In contrast, we were not able to elucidate exactly the pattern of reactivity for the other MAb, 1F6. MAb 1F6 did not react to the conserved 5-amino-acid stretch (S1) itself. In addition, changes in amino acid residues outside of the core stretch appeared to have a stronger effect on the reactivity of 1F6 than on that of 1B4.

There are 23 different types of clinically isolated NV strain capsid amino acid sequences in GenBank corresponding to the 11-amino-acid stretch recognized by the two MAbs. We examined the reactivities of MAbs 1B4 and 1F6 toward 19 different clinically isolated NV strains representing 19 of the 23 strain types in GenBank. Among these 19 stretches or recombinant capsid proteins of either GI or GII, 1B4 reacted to 18 and 1F6 reacted to 14. These results reflect the broad reactivities of the two MAbs toward NV capsid proteins.

The reactivities of the two MAbs to four strains newly deposited in GenBank (KU31GII, KU37aGII, KU37bGII, and KU80aGII; accession numbers AB058565, AB058570, AB058571, and AB058582, respectively) were not examined in this study. However, three of these four strains carry the conserved 5-amino-acid stretch. Thus, the possibility of the binding of MAb 1B4 can be hypothesized. These results suggested that MAbs 1B4 and 1F6 could be useful tools for detecting a broad range of NV strains present in clinical specimens.

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T. Yoda and Y. Suzuki contributed equally to this work.

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