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Expression, Self-Assembly, and Antigenicity of a Snow Mountain Agent-Like Calicivirus Capsid Protein

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Virus-like particles were produced in insect cells infected with a recombinant baculovirus containing the capsid gene of MX virus, a Mexican strain of human calicivirus. These recombinant MX (rMX) particles were morphologically similar to recombinant Norwalk virus (rNV) particles as observed under an electron microscope and contained a single capsid protein with a molecular weight of 57,000, which was slightly smaller than that of rNV. This protein was immunoprecipitated by sera from volunteers infected with the Snow Mountain agent, but it reacted weakly with sera from volunteers infected with NV. This protein did not react with hyperimmune antisera from animals immunized with rNV in the rNV antigen enzyme immunoassay (EIA). Seroresponses were detected from volunteers infected with Snow Mountain agent and Hawaii agent when the rMX particles were used as antigen in an EIA. This EIA also detected an immune response in the sera of child from whom the MX virus was isolated, and a high prevalence of antibody to MX virus was found in the sera of a cohort of Mexican children.

The recent molecular cloning and characterization of the Norwalk virus (NV) genome allowed NV to be classified as a member of the calicivirus (CV) family (6, 11). By using reverse transcription-PCR with primers designed from conserved regions of the NV genome, many viruses previously described as small round-structured viruses or human CVs (HuCVs) have been characterized (1, 7–9, 12, 13, 18, 19, 28). Sequence analysis of the amplified reverse transcription-PCR products showed that all of these viruses are related but that they are genetically diverse. By comparison of the sequences in the RNA-dependent RNA polymerase region, HuCVs can be divided into three groups: NV-like, Snow Mountain agent (SMA)-like, and HuCV/Sapporo-like (8, 18, 28). The antigenic relationships among these groups remain to be elucidated.

Expression of the NV capsid protein in baculovirus provided important reagents for use in the diagnosis of NV and NV-like virus infections. These recombinant NV (rNV) particles were indistinguishable antigenically and immunologically from NV found in clinical samples. New tests based on rNV viral particles were developed (3, 10, 23, 26), and new insights about the molecular characterization and epidemiology of HuCVs have come from application of the new tests (4, 14, 20, 24). However, the antigenic relationships of rNV with SMA-like viruses and other HuCVs remain to be fully described. A low level of cross-reaction between the prototypes of NV and SMA was detected by the rNV enzyme immunoassay (EIA) in serum specimens from volunteers infected with SMA (2, 26).

In our recent study of NV and NV-related viruses in Mexican children enrolled in a cohort study of diarrhea, we described the molecular cloning of MX virus (7). This strain was originally identified by reverse transcription-PCR in stool samples from a 6-month-old Mexican child with diarrhea. A >3-kb cDNA fragment was cloned. It extended from the RNA polymerase region to the 3′ poly(A) tail of the genome and included the viral capsid gene. Sequence analysis showed that MX virus had 91% amino acid identity with SMA, but only 60% amino acid identity with NV, in the RNA polymerase region (7). MX virus did not react with NV in the rNV antigen EIA. In this report we describe the characterization of the MX viral capsid as expressed in a baculovirus recombinant and demonstrate that the baculovirus-expressed MX viral capsid is antigenically different from the rNV-expressed capsid.

MATERIALS AND METHODS

Serum samples. Four pairs of serum samples from symptomatic volunteers infected with NV (3) were kindly supplied by Mary K. Estes. Five serum sample pairs from symptomatic volunteers infected with NV (one pair), SMA (two pairs), and the Hawaii agent (HA) (two pairs) were derived from previous studies (17) at the University of Rochester School of Medicine and Dentistry. A subset of 139 serum specimens collected sequentially from randomly selected Mexican children from birth to 2 years of age was included in the study (7, 27).

Baculovirus expression of rMX viral capsid protein. To construct a baculovirus-MX recombinant (rMX), a 2.4-kb DNA fragment from the 3.3-kb MX viral cDNA (7) containing open reading frame ORF2 and ORF3 of the MX viral genomes was excised and ligated into the baculovirus transfer vector pVL1392. In the recombinant plasmid pVLMX, the viral capsid protein gene is under the control of the polyhedron promoter. The orientation of the viral insert in the plasmid was confirmed by restriction enzyme and sequence analyses.

Plasmid pVLMX was cotransfected with wild-type baculovirus DNA into insect cells (*Spodoptera frugiperda* Sf9 cells) by procedures described previously (10). Recombinant viruses were obtained by three rounds of plaque purification. The plaque-purified rMX viruses were analyzed by PCR with primers specific for MX. Four clones which showed comparably high levels of expression of rMX viral capsid protein were selected, and viral stocks were prepared for expression experiments.

To express rMX capsid protein, Sf9 cells were infected with plaque-purified virus at a high multiplicity of infection (~10 units) and were harvested 5 to 6 days after infection. The supernatant and the cell lysates were pooled and pelleted for 2 h at 10,000 rpm in a Beckman J10 rotor. The resulting pellets were resuspended in 10 mM Tris (pH 7.4) and were extracted once with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113; also referred to as Genetron [Dupont Co., Wilmington, Del.]). The aqueous phase of the samples was loaded onto a discontinuous sucrose gradient (10 to 50%), and the mixture was centrifuged for 1 h at 25,000 rpm in a Beckman SW 28 rotor. The gradients were fractionated, and aliquots of each fraction were analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The peak fractions containing the viral capsid protein with a

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molecular weight of 57,000 were pooled, and the viral protein was then concentrated by pelleting for 2 h at 27,000 rpm in a Beckman SW28 rotor. The viral capsid proteins were further purified in a CsCl gradient at a density of 1.36 g/cm^3 and were centrifuged for 24 h at 35,000 rpm with a Beckman SW41 rotor. A visible band of the viral capsid proteins in the top third of the gradient was collected with a syringe. The CsCl was removed by dilution of the samples with water and pelleting for 1.5 h at 35,000 rpm in a Beckman SW41 rotor.

EIA to detect antibody against MX virus. To determine the antigenicity of rMX capsid, an EIA was developed to measure antibody in human sera by using the same format described elsewhere for the rNV antibody EIA (10). Highly purified rMX protein was used to coat microtiter plates (Dynatech) at a concentration of 1 μg/ml in 0.01 M phosphate-buffered saline (PBS; pH 7.4; 100 μl per well), and the plates were incubated overnight at 4°C. The antigen-coated microtiter plate was then blocked with 5% Blotto (Carnation nonfat milk) in PBS for 1 h at 37°C. After washing two times (200 μl per well) with 0.05% Tween 20–PBS (TW-PBS), serial dilutions of human serum samples (100 μl per well diluted in 1% Blotto–PBS) were added to the wells and the plate was incubated for 2 h at 37°C. After each well was washed six times with TW-PBS, the bound antibody was detected by adding horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG), IgA, and IgM (1:5,000 dilution in 1% Blotto-PBS; 100 μl per well; Cappel Organon Teknika, Durham, N.C.), and the plate was incubated for 2 h at 37°C. After a final washing six times with TW-PBS, the bound antibody was detected by the addition of TMB substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). After a 10-min incubation at room temperature, a solution of H_3PO_4 was added to stop color development. The reactivity of each well was quantitated at an optical density at 450 nm in a Titertek microtiter plate reader (Flow Laboratories, McLean, Va.).

RESULTS

Expression of MX viral capsid protein in a baculovirus recombinant. In our previous rNV baculovirus expression experiments, high yields of viral capsid protein were obtained from a recombinant baculovirus containing both ORF2 and ORF3 of the NV genome (10). A similar MX-baculovirus recombinant was constructed for expression of the rMX viral capsid protein. A major band of viral capsid protein was identified in the insect cells infected with rMX virus but not in the insect cells uninfected with the rMX virus (Fig. 1). This protein was slightly smaller than the rNV capsid protein in the polyacrylamide gel containing SDS and β-mercaptoethanol. The protein's apparent molecular weight of 57,000 suggested that this protein was derived from ORF2, not ORF3, of the MX viral genome.

The kinetics of rMX protein expression in insect cells were similar to those of rNV (10). The viral capsid proteins appeared at day 2 and reached a peak level at day 5 after infection (data not shown). However, in contrast to rNV, a higher proportion of the rMX viral capsid protein remained associated with the cell at the time of peak expression (data not shown). The protein obtained from the cell lysates did not shown any difference in size from that of the protein in the supernatant and was not associated with cellular proteins. Therefore, both the supernatant and the cell lysates were harvested. The method used to purify rNV capsid protein from the insect cells (10) was followed to purify the rMX capsid protein. A yield of 2 to 5 mg of purified rMX capsid protein per liter of culture was obtained.

The rMX viral capsid protein self-forms virus-like particles. Homogeneous virus-like particles were detected by electron microscopy in peak fractions of the protein with a molecular weight of 57,000 in both sucrose and CsCl gradients (data not shown). The CsCl density of the peak fractions of the viral capsid protein was 1.31 g/cm^3. Two types of particles were seen in the peak fractions. One type was indistinguishable from rNV particles, which were 30 to 35 nm in diameter, round, and empty, with 10 protruding points on the surfaces of the particles. The second particle type was smaller (15 to 20 nm in diameter), round, and empty, with six to eight points on the surfaces of the particles. Some of the small particles had a Star of David appearance. The small particles were seen less frequently in fresh preparations of the viral capsid proteins, but they were seen more frequently after the samples were stored at 4°C.

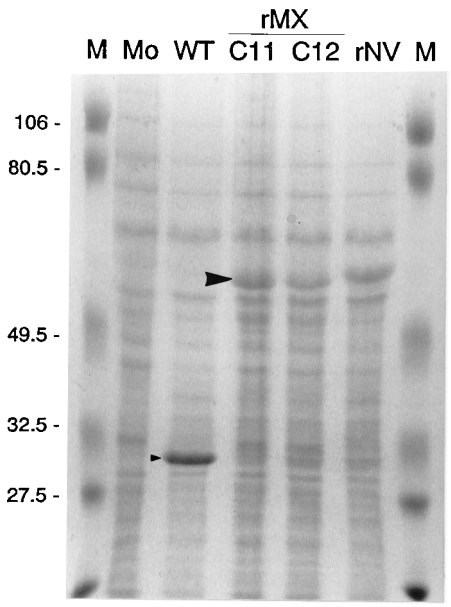

FIG. 1. Expression of rMX capsid protein in a baculovirus recombinant. Insect cells mock infected (Mo) or infected with rMX, wild-type (WT) baculovirus, or rNV were harvested at 5 days postinfection. Aliquots of the cell lysates and supernatants of the culture were electrophoresed in a 10% polyacrylamide gel and were stained with Coomassie blue. M, molecular weight marker (low-molecular-weight range; Bio-Rad); C5-11 and C5-12, individual clones of rMX recombinant viruses. The small arrowhead indicates the location of wild-type baculovirus polyhedron protein, and the large arrowhead indicates the location of rMX and rNV capsid proteins.

Antigenic relationship of rMX to prototype HuCVs. Three experiments were performed to determine the antigenicities of the rMX particles. We first tested the rMX particles in the rNV antigen EIA (3, 10). The purified rMX capsid protein was not detected by the rNV antigen EIA even at 2 μg of protein per ml. This result was consistent with our previous observation that MX viruses from human stool samples were not detected by the rNV antigen EIA (7).

Second, we tested the antigenicity of the rMX capsid protein with sera from volunteers infected with different prototype HuCVs. The rNV antigen also was included in each plate for comparison (10). Volunteers infected with SMA showed strong immune responses to rMX, but they showed a weak or no response to rNV (Table 1). Similar immune responses were observed for two volunteers infected with HA. In contrast, five volunteers infected with NV showed a weak response (4- to 16-fold) or no response to rMX, responses distinctly less than those to rNV that were observed.

The specificity of the results described above was confirmed by a blocking experiment in which serum specimens from the volunteers were preincubated with one of the two antigens before they were added to wells. Specific blocking was observed by homologous antigens, but no or marginal blocking was observed by heterologous antigens (Fig. 2). Antibodies to rMX antigen in sera from patients infected with SMA and HA, respectively, were both blocked by rMX, but not by rNV, suggesting that SMA and HA share common antigenic epitopes, which are not shared with NV, on the surfaces of the viral particles.

TABLE 1. Serological responses to rMX and rNV among volunteers infected with SMA, HA, or NV

Infecting agent	Volunteer	Antibody titer prechallenge/postchallenge (fold change):	
		rMX	rNV
SMA	86-25	1,600/102,400 (64)	400/1,600 (4)
	86-28	1,600/25,600 (16)	100/400 (4)
HA	86-33	1,600/25,600 (16)	400/400 (0)
	86-38	1,600/102,400 (64)	400/400 (0)
NV	523	1,600/6,400 (4)	1,600/102,400 (64)
	529	1,600/6,400 (4)	400/25,600 (64)
	544	6,400/6,400 (0)	400/25,600 (64)
	543	1,600/25,600 (16)	400/25,600 (64)
	77-02	6,400/25,600 (4)	6,400/102,400 (16)

Third, we tested the rMX protein by immunoprecipitation with sera from volunteers infected with SMA or NV. The viral capsid protein was specifically precipitated by postinfection sera but weakly by preinfection sera from volunteers infected with SMA. This protein also was precipitated by postinfection sera from volunteers infected with NV. The protein with a molecular weight of 34,000 found in rNV-infected cells (10) was not detected.

Detection of serological response to rMX in Mexican children. The rMX EIA described above was optimized for detection of antibody to MX virus in serum specimens from Mexican children. First, we tested the serum specimens from the child from whom we cloned the MX virus. A continuous increase in antibody titers to rMX virus was observed in serial specimens from the child from birth to 18 months of age. A significant increase (titers, 1:100 at birth to 1:1,024 at age 8 months) was noticed after the diarrhea episode in which the MX virus was detected. In another set of 139 specimens from 20 children in the same cohort, 63% had antibodies to rMX in the first week of life, 31% had antibodies at ages 4 to 6 months, 78% had antibodies at ages 10 to 12 months, and 100% had antibodies at ages 22 to 24 months.

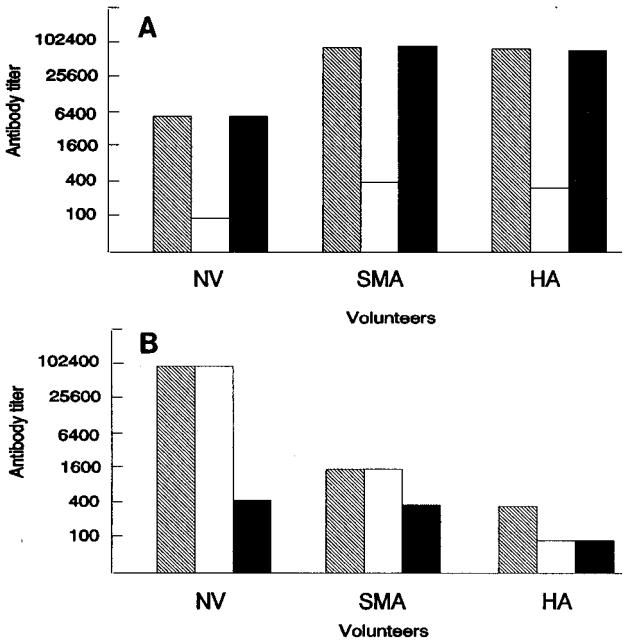

FIG. 2. Determination of cross-reactivity between rMX and rNV in blocking experiments. Serum specimens from volunteers infected with NV, SMA, and HA were titrated for antibody to rMX (A) and rNV (B) by EIAs. Serial dilutions of each serum sample were tested directly or after preincubation with rMX or rNV antigens. The three bars represent no blocking, blocking with rMX, or blocking with rNV from left to right, respectively.

DISCUSSION

We previously described the molecular characterization of MX virus, a SMA-like HuCV, from a child in our prospective study of diarrhea in Mexico (7). This report described the expression, morphology, and antigenicity of the rMX viral capsid protein in the baculovirus system. This is the second HuCV capsid expressed in the baculovirus system; the first one was rNV (10). The unlimited supply of rMX particles will permit development of diagnostic assays to further study the immunology and epidemiology of HuCVs.

We have demonstrated that the rMX particles are similar morphologically but are distinct antigenically from rNV, confirming our hypothesis that MX and NV belong to different antigenic groups. We also demonstrated that the rMX EIA detects strong immune responses in volunteers infected with SMA and HA, providing evidence that MX virus, SMA, and HA should be grouped in the SMA-like antigenic group, as was suggested by the genogrouping (8, 28). The rNV particles detected strong serological responses in volunteers infected with NV but not in volunteers infected with SMA and HA. Therefore, serological responses detected by EIAs with rMX or rNV particles likely can be used for the diagnosis of SMA-like and NV-like virus infections, although both antigens may need to be used to interpret the meaning of the responses detected to one of them.

We observed a low level of cross responses in volunteers infected with NV, SMA, and HA by the rMX and rNV EIAs, but we also demonstrated that the antibodies detected by the two EIAs were against different epitopes. The low levels of cross responses detected in the volunteers may be influenced by their exposure histories. If the volunteers were exposed previously to MX virus or NV, a strong antibody response may occur on reexposure, but this infection also may stimulate a low-level antibody response to closely related antigens. This type of cross response was not found in the hyperimmune antisera to rNV obtained from animals (7). The detection of these cross responses when using purified antigens indicates the limits of previous results which characterized the antigenic relationships among HuCVs (15, 16, 22) with human reagents.

The rMX and rNV capsids have similar sizes, morphologies, and particle densities. The finding of small subviral particles as well as complete particles in the peak fractions of the CsCl gradient of both the rMX and rNV capsids (5a) suggested that the small particles may be components of the capsids. Similar small particles also have been observed in human stool samples containing typical HuCV particles (17a, 25). The finding of more small particles than complete particles in the stored samples indicated that the small particles may be a degradation product from complete particles. Additional experiments to determine the biochemical and biophysical properties of the small particles are required to understand their biological significance.

Differences between rMX and rNV capsids were also found. First, the predicted gene product of ORF2 of MX virus is larger (19 amino acids) than that of NV, but the MX viral capsid is smaller when the two expressed capsids were compared by gel electrophoresis. In addition, the small soluble

protein with a molecular weight of 34,000 found in the rNV-infected insect cells was not seen in rMX-infected cells. Proteins with molecular weights of 63,000 or 33,000 proteins have been detected in clinical specimens containing NV-like viruses in variable amounts (5, 21). It is interesting that one strain of a small round-structured virus (Oth-25) which contained only the protein with a molecular weight of 33,000 (21) was genetically very close to MX virus (7, 28). Further experiments are required to determine whether the small protein is another form of the viral capsid or a degradation product of the full-length viral capsid.

The serological studies indicate that MX virus was the causative strain of the diarrhea episode in the Mexican child from whom it was isolated and that antibody to MX virus was acquired at an early age among the Mexican children within our cohort study. Further studies with MX virus-specific reagents will permit comparative study of differences in the epidemiology of HuCVs in the NV-like and SMA-like genogroups.

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