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Expression of the Hantaan Virus M Genome Segment by Using a Vaccinia Virus Recombinant

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A cDNA containing the complete open reading frame of the Hantaan virus (HTN) M genome segment has been cloned into vaccinia virus. This recombinant virus expresses two glycoproteins which are similar to the HTN structural glycoproteins, G1 and G2, in molecular weight, cleavage pattern, and cellular distribution. Both HTN and recombinant vaccinia virus glycoproteins are exclusively associated with the Golgi apparatus of the cell. Despite this intracellular restriction, mice inoculated with the recombinant vaccinia virus raised neutralizing antibodies against HTN. The specificity of virus neutralization appears to reside in the HTN glycoproteins, since a vaccinia virus recombinant expressing the HTN nucleocapsid protein was unable to elicit a neutralizing antibody response.

Hemorrhagic fever with renal syndrome, a group of clinically similar syndromes affecting humans, includes Korean hemorrhagic fever, hemorrhagic nephroso-nephritis, nephropathia epidemica, and epidemic hemorrhagic fever (EHF). Cases of hemorrhagic fever with renal syndrome were originally reported in Korea, Scandinavia, Europe, the Soviet Union, and China (24). Hantaan virus (HTN) was discovered 10 years ago by Lee and Johnson as the etiologic agent of Korean hemorrhagic fever (10). They demonstrated an antigen in the lungs of the wild striped field mouse, *Apodemus agrarius*, which gave specific immunofluorescent reactions with serum samples from convalescent hemorrhagic fever patients. Since that time, numerous viruses such as Puumala virus, Prospect Hill virus, Lee virus, and other antigenically related Hantaan-like viruses have been implicated in hemorrhagic fever with renal syndrome-type diseases (11, 23). Recently, seroconversion of human populations to Hantaan-related viruses have been demonstrated in almost every part of the world, including the United States (4). As a result of biochemical and molecular characterization of HTN over the past few years, it has become apparent that HTN should be classified as a separate genus of the *Bunyaviridae* family (22). Recently, the International Committee on the Taxonomy of Viruses has designated HTN as the prototype species of a new and separate genus called the *Hantavirus* genus (26).

Like other members of the family *Bunyaviridae*, HTN has a tripartite, single-stranded, negative-polarity RNA genome, in which the three segments L, M, and S have molecular masses of about 2.7, 1.2, and 0.6 megadaltons (MDa), respectively (24). The viral envelope contains two virus-specific glycoproteins, G1 and G2, with respective molecular masses of approximately 65 and 55 kDa (6). The three circular viral nucleocapsids are associated with the 50-kDa viral nucleocapsid protein, N (6).

Recently, both the M and S genome segments of HTN were cloned as cDNAs and the nucleotide sequences were determined. Sequence data generated from the HTN S genome segment suggests that the HTN N protein may be the exclusive product of that RNA segment (26). Cell-free translation of RNA generated from the S-segment cDNA further supports this hypothesis (26). Sequence data generated from the HTN M genome segment revealed a single open reading frame which has the potential to encode a polypeptide of 126 kDa. Amino-terminal sequencing of isolated G1 and G2 glycoproteins was performed and revealed a gene order with respect to message sense RNA of 5′-G1-G2-3′ (27). Reports from those who have sequenced the M genome segment of other *Bunyaviridae* members show an analogous large open reading frame with the capacity to encode a polyprotein which can presumably be processed into the envelope glycoproteins; however, the gene order has not been determined in every case (7, 8, 12). Therefore, it appears that members of the family *Bunyaviridae* utilize a common strategy in assignment of genes to genomic segments. However, much remains to be worked out in determining the steps involved in the production of mature glycoproteins from the M-segment mRNA.

In this paper we report initial studies on the use of vaccinia virus as an expression vector system to study the production of the glycoproteins from the HTN M genome segment. We have chosen vaccinia virus as an expression system because of the numerous reports that recombinant vaccinia virus-infected cells express high levels of the recombinant protein with proper processing, glycosylation, and cellular transport (13–15, 18, 21). In this report we demonstrate that, indeed, recombinant vaccinia virus is a useful tool in the study of HTN expression. We clearly show that the M-segment cDNA, cloned into vaccinia virus, produces two glycoproteins, G1 and G2. These proteins are similar to authentic HTN G1 and G2 glycoproteins in molecular weight, antigenic reactivity with specific sera, and association with the Golgi apparatus of the cell. Despite the restricted intracellular location of these two glycoproteins, we also show that vaccinia virus recombinants containing the M genome segment raise neutralizing antibodies in mice, whereas vaccinia virus recombinants expressing the S segment (nucleocapsid protein) do not.

MATERIALS AND METHODS

Cells, virus, and infections. Vaccinia virus WR and vaccinia virus recombinants vSC8, vMP2, and v1009 were propagated in CV-1 cells at 37°C in Eagle minimum essential

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medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% (vol/vol) fetal calf serum and 10 μg of gentamicin sulfate per ml. Human 143 TK^- cells were maintained in similar medium supplemented with 5'-bromodeoxyuridine at 25 μg/ml. Crude vaccinia virus was harvested from infected cells after 2 days of infection by collecting cells and subsequent freeze-thawing. Gradient-purified virus was prepared as required, as described by Mackett et al. (14). HTN 76-118 (a strain previously adapted to Vero E6 cells) was grown as previously described (24).

Construction of vaccinia virus recombinants. All DNA and plasmid manipulations were carried out as described by Maniatis et al. (16). The vaccinia virus insertion vector pSC11 was generously provided by B. Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Restriction enzymes, T4 DNA ligase, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. T4 DNA polymerase was purchased from New England BioLabs, Inc., Beverly, Mass. The construction of pMP1, a pSC11 recombinant plasmid containing the entire M56 fragment, is described in the legend to Fig. 1. Recombinant vaccinia virus was prepared by transfecting subconfluent vaccinia virus WR-infected CV-1 monolayers with pMP1 by the calcium P_i method (14). TK^- recombinants were then isolated by a plaque assay of transfected-cell lysate on 143 TK^- cells with 1% low-melting-point agarose overlay containing 25 μg of bromodeoxyuridine per ml and 300 μg of X-Gal per ml. Blue plaques were removed and subjected to three rounds of plaque purification. The DNA isolated from the recombinant virus was examined by restriction endonuclease digestion and Southern blot analysis to confirm the predicted insert.

Immunoprecipitation and antisera. Cells were infected with all vaccinia virus recombinants at a multiplicity of infection of 10 and with HTN at a multiplicity of infection of 0.1 to 1.0. Intracellular viral proteins were pulse-labeled with [^{35}S]methionine (100 μCi/ml) at 24 h postinfection. Before being labeled, cells were maintained in medium without methionine for 1 h then pulsed for 4 h. They were then washed three times in ice-cold phosphate-buffered saline (PBS) and then lysed in 1 ml of mRSB buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–KOH (pH 7.4), 1.5 mM $MgCl_2$, 20 mM KCl) containing 1% (vol/vol) Nonidet P-40, 1% (vol/vol) aprotinin (Sigma Chemical Co., St. Louis, Mo.), and 10 μg of alpha-2-macroglobulin (Sigma) per ml. Cell nuclei were removed by centrifugation at 12,000 × *g* for 5 min at 4°C. Cell lysate (supernatant) (100 μl) was diluted with 1 ml of mRSB buffer and appropriate antisera (5 to 10 μl) and kept overnight at 4°C. This procedure was followed by the addition of 100 μl of 10% (wt/vol) protein A–Sepharose (Sigma) for 1 h at 4°C with end-over-end agitation. Precipitates were washed three times in mRSB buffer and then suspended in 150 μl of 1× sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 2.3% [wt/vol] sodium dodecyl sulfate, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol) for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal rabbit antisera consisted of convalescent-phase serum samples collected 4 to 12 weeks following infection of New Zealand White rabbits with HTN (strain 76-118). A monoclonal antiserum, HCO2, and an antipeptide serum, 60411, were used as previously described (27). Both are specific for G2. Antipeptide serum 60411 was raised from a peptide that extends from amino acids 1127 through 1135 of G2. All antisera were preadsorbed with acetone-fixed vSC8-infected CV-1 cells prior to use for immunoprecipitation or immunofluorescence.

Indirect immunofluorescence. Subconfluent monolayers of CV-1 cells were grown on glass cover slips and infected with vaccinia virus recombinants as shown in Fig. 4. At various times after infection, cells were washed three times in ice-cold PBS and immediately fixed in acetone at −20°C for 10 min. Cover slips were then rehydrated in PBS containing 10% (wt/vol) FCS for 15 min at room temperature. Following this, they were incubated with an appropriate dilution of antibody for 1 h and then washed three times in PBS. Cells were then exposed to goat anti-mouse immunoglobulin G antibody conjugated with rhodamine (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h and washed three times with PBS. The cover slips were then mounted in 50% glycerol and viewed with a phase-fluorescence microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

Animal experiments. Duplicate groups of five 5-week-old BALB/CByJ mice (Jackson Laboratory, Bar Harbor, Maine) were vaccinated with the appropriate virus by tail scarification. Blood was collected from the retro-orbital sinus of each mouse at 2, 4, and 6 weeks post-vaccination. Manipulations with infected animals were performed under P-3 containment conditions.

Virus neutralization. Plaque reduction neutralization tests were performed on CV-1 (ATCC CCL70) monolayers grown in Eagle minimal essential medium as previously described (23). Specific mouse sera, serially diluted twofold, were incubated overnight at 4°C with 40 to 50 PFU of HTN 76-118. All dilutions were done with medium containing 2% (vol/vol) fresh monkey serum. Inoculated cells were further incubated for 1 h at 37°C before the addition of an overlay containing 0.6% (wt/vol) agarose. Plaques were stained and read after 7 days. Titers were reported as 50% or 80% plaque reduction neutralization titers, which represent the reciprocal of the highest dilution of antibody resulting in more than 50% or 80% reduction of plaques, respectively. The titer of each mouse serum was found individually, and an average serum neutralization titer was determined by geometrically averaging the results for all animals given similar vaccinations.

RESULTS

Construction of a vaccinia virus recombinant containing the M genome segment of HTN. The generation of the M56 plasmid has been previously described (27). Briefly, M56 is a cDNA clone of the HTN M segment which contains the entire M RNA segment, except for 6 bases corresponding to the 3' terminus and 3 bases corresponding to the 5' terminus of the virion RNA. Within the one major open reading frame, protein synthesis could potentially initiate at one of two in-frame AUGs located at nucleotide positions 41 to 43 or 65 to 67 and terminate at a stop codon at positions 3446 to 3448. Therefore, even though M56 is not a complete cDNA, it contains all the information necessary to code for the products of the M genome segment. We chose to use plasmid pSC11 as the vaccinia virus insertion vector (2), since pSC11 has a single convenient *Sma*I site just downstream of the early-late vaccinia virus promoter P7.5, as well as β-galactosidase expression for ease of selection. Because there are no ATG translational initiation codons between the transcriptional start site and the insertion site, translation of RNA transcribed from a gene(s) under P7.5 regulation is dependent upon an AUG provided by the foreign gene. In this case, either of the two M56 initiation codons would be

potentially functional. As outlined above, pSC11 also contains an *E. coli lacZ* gene under the control of a second vaccinia virus promoter, P11, which allows for the identification of recombinant virus through the appearance of blue plaques upon addition of X-Gal to the agarose overlay. Both the P7.5 promoter and the P11 β-galactosidase transcriptional unit are flanked by vaccinia virus TK sequences for homologous recombination.

We inserted the M56 fragment into pSC11 such that the 3′ genomic end was proximal to the P7.5 promoter (Fig. 1). This orientation allows for transcription of message-sense M-segment RNA to begin from the P7.5 promoter and terminate in the TK transcription-termination region. The resultant hybrid insertion plasmid was designated pMP1. Using this construct recombinant, we prepared vaccinia virus and isolated the resultant blue plaques. Crude DNA

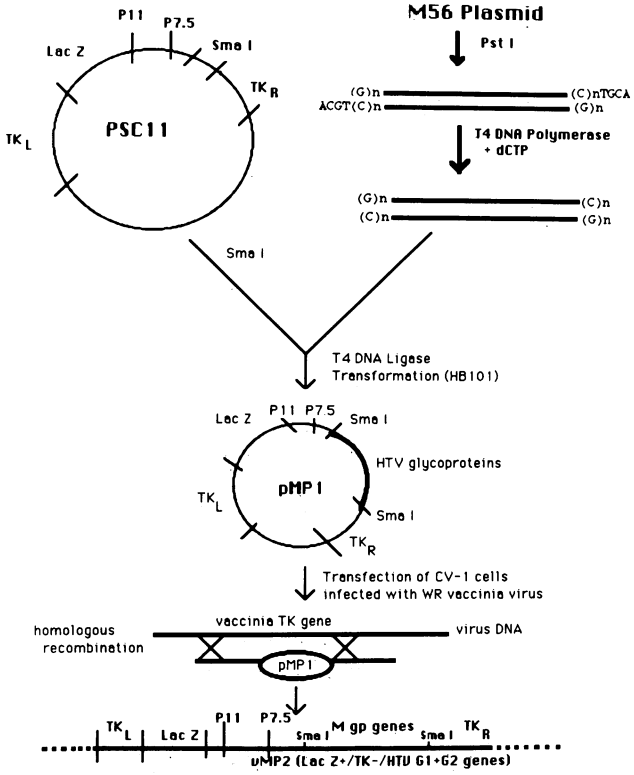

FIG. 1. Cloning strategy used to generate vMP2, a recombinant vaccinia virus containing the entire open reading frame of the M genome segment of HTN. The M56 fragment was isolated from a *Pst*I digest of M56 plasmid. The 3′ *Pst*I overhangs were removed by digestion with T4 DNA polymerase, and the blunt-ended fragment was cloned into the *Sma*I site of pSC11 to generate the recombinant insertion vector pMP1. pMP1 was transfected into CV-1 cells already infected with wild-type vaccinia virus (WR strain). After 48 h, the transfection supernatant was removed and screened for Lac^+ TK^- recombinant vaccinia virus by the production of blue plaques on human 143 TK^- cells grown in the presence of bromodeoxyuridine and X-Gal. A slightly different strategy was used to generate v1009 (a vaccinia virus recombinant containing the entire S coding sequences). The S-86 fragment was isolated from plasmid S-86 (26) and subcloned into the *Pst*I site of pUC9. This recombinant was then digested with *Sma*I and partially digested with *Hin*dIII. The resultant S-86 fragment was made blunt ended by filling in the 5′ *Hin*dIII overhang with Klenow polymerase and was subsequently cloned into the *Sma*I site of pSC11. Recombinant virus was obtained by the same method as described for vMP2.

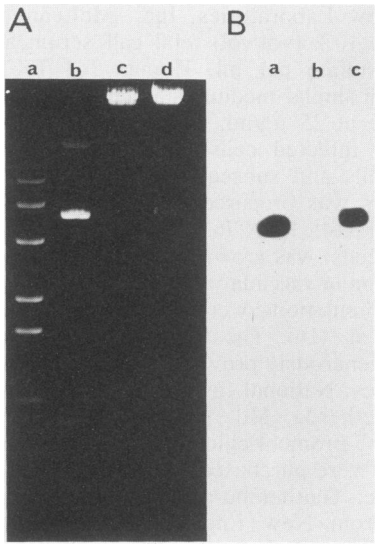

FIG. 2. Analysis of DNA from the vMP2 recombinant. (A) Ethidium bromide stain of *Sma*I-digested DNA electrophoresed on a 0.8% agarose gel. Lanes: a, 1 μg of a 1-kilobase molecular size ladder supplied by Bethesda Research Laboratories; b, 1.5 μg of the hybrid insertion vector pMP1; c and d, 1.5 μg of DNA from gradient-purified vaccinia virus recombinants vSC8 and vMP2, respectively. (B) Southern blot. Lanes a, b, and c of panel B correspond to lanes b, c, and d, respectively, of panel A. Gel-purified ^{32}P-labeled M56 fragment was used as a probe.

was isolated from cells infected with several different blue plaque isolates. Dot blot analysis revealed several positive recombinants upon probing with a ^{32}P-labeled M56 fragment. One of these dot blot positive vaccinia virus-HTN recombinants, designated vMP2, was used for further characterization.

Characterization of vMP2. The structure of the genome of vMP2 was analyzed by gradient purification of virus, extraction of the viral DNA, and subsequent restriction endonuclease digestion. We chose to use the vaccinia virus recombinant vSC8 as a negative control throughout our studies. vSC8 is a recombinant vaccinia virus generated from the plasmid pSC8; it has a TK^- phenotype, contains the *lacZ* gene under the P11 promoter, but contains no other foreign DNA (2). Digestion of the 3′ *Pst*I overhangs of the homopolymer-tailed M56 fragment with T4 DNA polymerase has created a convenient *Sma*I site when cloned into the *Sma*I site of pSC11 (Fig. 1). In light of this, and because vaccinia virus DNA is A+T rich, we digested the isolated vaccinia virus DNA with *Sma*I. Figure 2A shows an ethidium bromide stain pattern of vMP2, vSC8, and pMP1 DNA after *Sma*I digestion. As expected, only vMP2 and pMP1 show the appearance of a single DNA band of 3.65 kilobases corresponding to the M cDNA fragment of M56. It should be noted that the HTN DNA insert in vMP2 migrates slightly slower than that from the vaccinia virus insertion vector from which it was derived. We attribute this difference to a gel artifact rather than an aberrant recombinational event, although the latter possibility cannot be ruled out at present. The DNA from the agarose gel was then transferred to nitrocellulose and probed with ^{32}P-labeled M56 fragment. The recombinant virus vMP2 contains the M56 cDNA fragment of predicted size and specificity, and DNA isolated from vSC8 does not react with the specific cDNA probe used (Fig. 2B). Northern (RNA) blot analysis of RNA extracted

from vMP2-infected cells also showed M56-specific mRNA in vMP2 infection (3.7 kilobases), but not in vSC8- or mock-infected cells (data not shown).

Identification of proteins produced from HTN-vaccinia virus recombinants. To examine the HTN-specific proteins expressed by vMP2, Vero E6 cells were infected with HTN, vSC8, vMP2 or mock-infected and pulse-labeled with [^{35}S]methionine after 24 h of infection. The radioactively labeled proteins were immunoprecipitated with a polyclonal, polyvalent anti-HTN rabbit antiserum. HTN-infected cells exhibited a protein pattern similar to that of mock-infected cells (Fig. 3A, lanes a and b). It has been known for some time that HTN causes no discernable cytopathic effect in infected cells, which may be related to its inability to shut off host protein synthesis. In contrast, polyacrylamide gel electrophoresis patterns for the vaccinia virus-infected cells showed a complete replacement of host proteins with that of vaccinia virus (Fig. 3A, lanes c and d). However, lysates from HTN-infected cells reacted with the antiserum to yield three predominant bands corresponding to the G1, G2, and N proteins (Fig. 3A, lane f), whereas vMP2-infected cell lysates reacted with the same antiserum to give proteins that appeared to comigrate only with HTN G1 and G2 (Fig. 3A, lane g). We also observed two high-molecular-weight proteins in Fig. 3A, lane g. We believe the larger of the two proteins to be β-galactosidase, as evidenced by its presence in vSC8-infected cells (Fig. 3A, lane h) and absence in wild-type vaccinia virus- and mock-infected cells. The additional high-molecular-weight protein seen specifically in lane g may be a possible polyprotein precursor. However, appearance of this band is quite variable, and positive confirmation would require more extensive immunological and biochemical analyses. G1 and G2 are also made in CV-1 cells infected with vMP2 (Fig. 3B, lanes b and d). However, the G1 synthesized appears larger and more heterogeneous than that seen in the Vero E6 cells. These results suggest that the HTN G1 and G2 glycoproteins produced from the vaccinia virus recombinant vMP2 have been cleaved and processed correctly in the two cell lines to which HTN has been adapted (24).

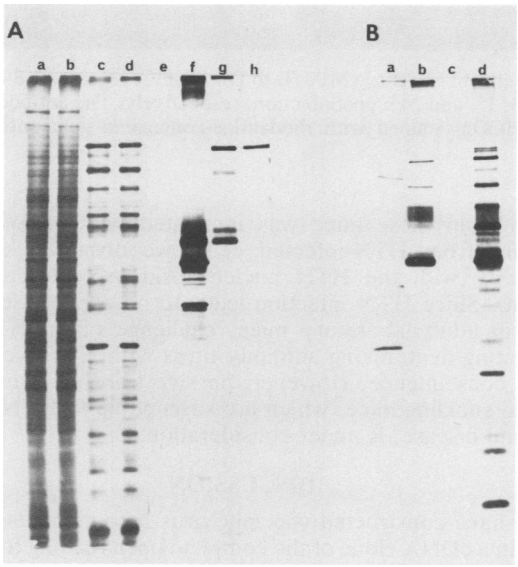

FIG. 3. (A) Polyacrylamide gel analysis of [^{35}S]methionine-labeled proteins from lysates of Vero E6 cells infected with vMP2 (lanes c and g), vSC8 (lanes d and h), or HTN (lanes b and f) or mock infected (lanes a and e). Lanes a through d represent 5% of the total starting material used for the immunoprecipitations shown in lanes e through h, respectively. The antibody used for the immune precipitations was a polyclonal anti-HTN rabbit antiserum. Relative molecular weights were determined by electrophoresis of [^{14}C]labeled high-molecular-weight standards obtained from Bethesda Research Laboratories (markers not shown). (B) Polyacrylamide gel analysis of [^{35}S]methionine-labeled proteins immunoprecipitated from lysates of CV-1 cells infected with vMP2 (lanes b and d) or vSC8 (lane c) or mock infected (lane a). Conditions and antiserum for lanes a to c were identical to those described in panel A. The antiserum used in lane d is the G2 antiserum 60411.

Expression of the glycoproteins from HTN-vaccinia virus recombinants. Members of the family *Bunyaviridae* differ from other negative-stranded enveloped RNA viruses in their site of budding, which takes place in the endoplasmic reticulum and Golgi apparatus, but not at the plasma membrane (5). Indirect immunofluorescence of HTN-infected CV-1 cells stained with antisera directed against G1 and G2 shows that both glycoproteins are exclusively associated with the Golgi apparatus and that no antigen is seen either on the plasma membrane or in the nucleus (Fig. 4A). We therefore decided to use this assay as a means of verifying that the vaccinia virus recombinant vMP2 was expressing HTN glycoproteins which could be both correctly processed and transported within the cell.

CV-1 cells were infected with vSC8 or vMP2 or mock infected, acetone fixed, and examined with a monoclonal antibody specific for G2 or polyclonal rabbit antibody reactive with G1 and G2 (see Materials and Methods). Both antisera gave similar results. Figure 4A shows the HTN-infected cells, and Fig. 4B to D show the immunofluorescence pattern obtained during the course of infection with vMP2. No fluorescence was seen in vSC8- or mock-infected cells (data not shown). We began to observe the appearance of G1 and G2 within 3 h postinfection and have found that expression continues and is maintained at high levels up to 48 h postinfection.

When comparing Fig. 4A with Fig. 4B to D, one must keep in mind that HTN-infected cells did not show any cytopathic effect, whereas a cytopathic effect was quite evident in the vaccinia virus-infected cells within 4 h postinfection; this may account for the apparent morphological differences between the two cell populations. Basically, in both cases the immunolocalization appears specifically in the perinuclear regions of the cell. This type of immunofluorescence pattern has been observed with other proteins, including the coronavirus E1 glycoprotein. Histochemical analysis with nucleoside diphosphatase activity as a marker Golgi enzyme confirmed the cellular localization of E1 as being the Golgi apparatus (5). By extrapolation, we believe that although histochemical analysis has not been performed, G1 and G2 show fluorescence staining strongly indicative of Golgi association. In striking contrast to the immunofluorescence patterns observed with vMP2-infected cells, we have recently found that a vaccinia virus recombinant containing the HTN S genome segment (v1009) shows multiple bright fluorescence spicules throughout the cytoplasm. However, as is the case with vMP2, this construct does not express HTN antigen on the cell surface or in the nucleus (manuscript in preparation).

Immunological response in BALB/c mice. We were interested in ascertaining whether mice vaccinated with vMP2 raised neutralizing antibodies against HTN. Since G1 and G2, both in HTN- and vMP2-infected cells, appear exclusively Golgi associated rather than being exposed on the cell surface, it was quite possible that mice infected with these

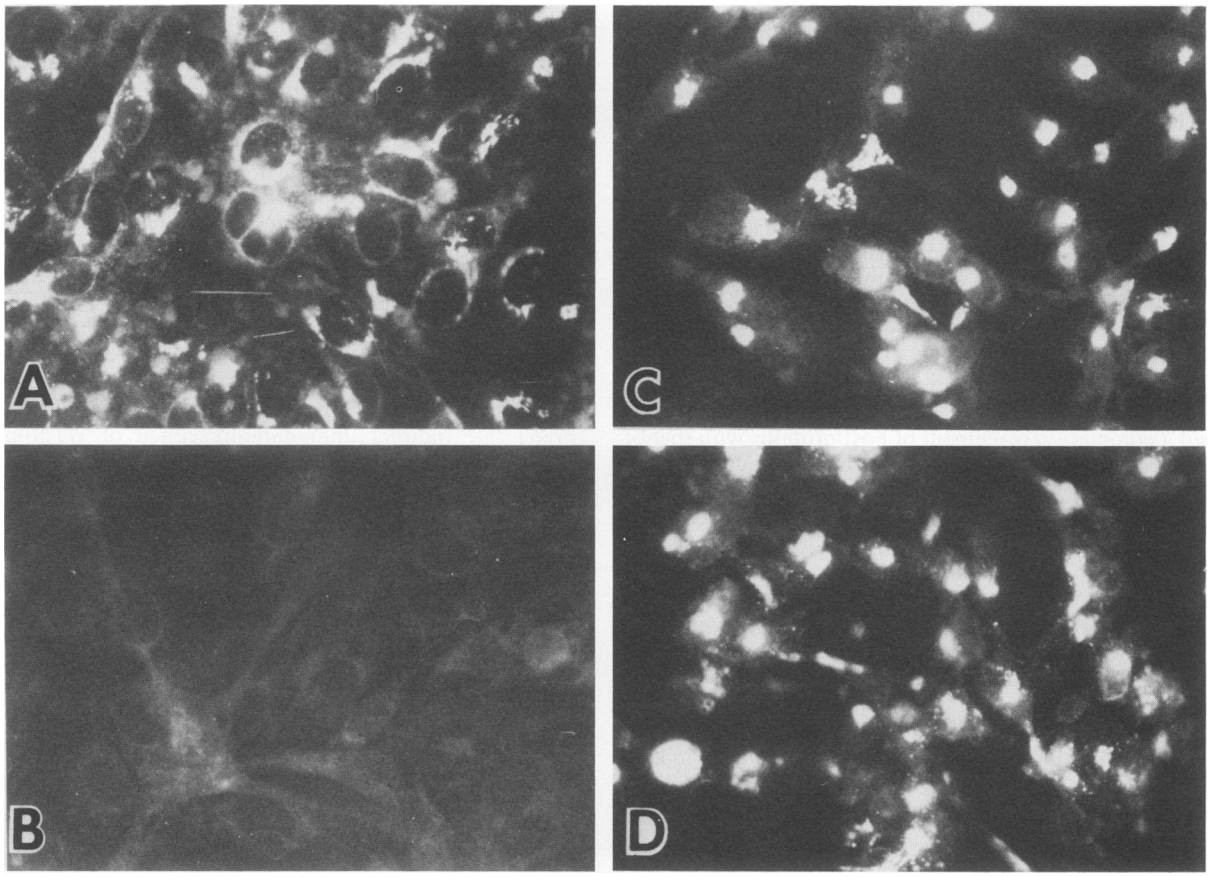

FIG. 4. Fluorescent-antibody labeling of acetone-fixed CV-1 cells infected with HTN (A) or vMP2 (B to D). Panel A represents 8-day-old HTN-infected cells. Panels B, C, and D represent cells infected with vMP2 at 3, 15, and 24 h postinfection, respectively. The antibody used was a monoclonal anti-HTN antibody against the HTN G2 glycoprotein (HCO2) stained with rhodamine-conjugated goat anti-mouse immunoglobulin G antiserum. Magnification, ×396.

viruses would raise only low levels of circulating antibodies against these proteins. Mice were inoculated by tail scarification with vMP2, vSC8, v1009 or medium alone and bled at 2-week intervals. After the second bleed, mice were boosted with an equivalent dose of virus. Tail scarification of mice with vMP2 results in significant neutralizing antibody titers against HTN after 2 weeks; subsequent boosts did not seem to have any significant effect (Table 1). In comparison, vaccination with v1009 (containing the HTN nucleocapsid protein) did not result in the production in serum of any neutralizing antibodies against HTN. However, when antiserum from these mice was incubated with radiolabeled proteins from HTN-infected cells, we observed specific reactivity with the HTN nucleocapsid protein (data not shown). Since HTN infection leads to no apparent disease state in adult laboratory mice, challenge of mice having circulating neutralizing antibody titers with HTN would be of no consequence. However, passive transfer of immune sera to suckling mice, which are susceptible to HTN infection and disease, is under consideration.

TABLE 1. Average neutralization titers in serum samples from mice vaccinated with recombinant vaccinia virus containing either the M or the S genome segment from HTN

Immunization	Antibody titer[a] on day:					
	14		28		42[b]	
	50%	80%	50%	80%	50%	80%
vMP2	640	300	700	320	740	380
vSC8	<20	<20	<20	<20	<20	<20
v1009	<20	<20	<20	<20	<20	<20
Medium	<20	<20	<20	<20	<20	<20

[a] Titers represent the reciprocal of the highest dilution of serum giving a reduction of plaques greater than 50% or 80%.
[b] Represents the titer from mice receiving a booster vaccination on day 28.

DISCUSSION

We have constructed vaccinia virus recombinants which contain a cDNA clone of the complete open reading frame of the M genome segment RNA of HTN, the prototype virus of the *Hantavirus* genus of the *Bunyaviridae* family. On the basis of reactivity with anti-HTN antisera, the ability to elicit virus-neutralizing antibodies, and similar cellular localization to that of HTN infection, we show that the vaccinia virus construct vMP2 produces two glycoproteins that are functionally similar to the HTN glycoproteins G1 and G2. It would, however, be premature to state that the proteins expressed by the recombinant are antigenically identical to the native proteins without performing an extensive characterization with a battery of monoclonal antibodies directed specifically against G1 or G2. In that context, it is apparent that the G1 produced in CV-1 cells has a larger M_r and is more heterogeneous than that seen in Vero E6 cells (Fig.

3B). This variation between the two cell types is probably due to differences in glycosylation: G1 has five potential asparagine-linked glycosylation sites, whereas G2 has only two (25, 27). Similar variations in other glycoproteins have also been reported. For example, Pereira et al. (19) have shown that herpes simplex virus type 1 and 2 glycoproteins gA and gB, when grown in Vero cells, had apparent molecular masses of 121 and 123 kDa, respectively, whereas glycoproteins gA and gB produced in HEp-2 cells had apparent molecular masses of 130 and 133 kDa, respectively. Despite these differences in G1, our findings clearly support the sequence data reported by Schmaljohn et al. (27) and are in agreement with the gene strategy used by other members of the *Bunyaviridae* family (7, 8, 12).

Having demonstrated that this recombinant virus encodes two antigenically functional HTN proteins, we can now study the molecular interactions of these proteins without having to use infection with HTN. This offers a unique advantage, since HTN represents a significant biohazard and has been assigned to a Biosafety Level 3 Containment in the U.S. Public Health Service guidelines (6). In addition, studies on the molecular biology of HTN genes are limited by the slow growth of the virus in cell culture. Maximum virus titer is not achieved until 8 days postinfection, and virus yields are typically no better than 10^6 PFU/ml. These data, taken together, suggest that HTN growth is characterized by persistent infection with a low steady-state level of viral protein in the infected cell. Host cell protein synthesis inhibition is not apparent (Fig. 3A).

Infection with a vaccinia virus recombinant offers several advantages in studying the processing of the HTN glycoproteins. Indirect immunofluorescence studies indicate that the vaccinia virus proteins remain associated with the Golgi apparatus in a manner analogous to HTN G1 and G2. This suggests that the M-segment sequences specify all of the necessary signals for processing and cleavage; in addition, these cleavages can apparently occur in the absence of HTN RNA replication or expression of other HTN proteins such as the nucleocapsid protein. We have demonstrated that over the course of infection, the recombinant proteins can accumulate in the infected cell up to 48 h postinfection. Therefore, we believe that this system is ideally suited for the use of site-directed mutagenesis in the study of the sequences necessary for the processing, transport, and immunogenicity of the HTN glycoproteins. Similar types of studies involving the use of a vaccinia virus recombinant have been recently performed on the rotavirus VP7 glycoprotein. Andrew et al. (1) have shown that cloning a genetically altered cDNA, containing a deletion of an N-terminal hydrophobic anchoring domain, into vaccinia virus results in the secretion of the normally rough-endoplasmic-reticulum-associated viral glycoprotein. We have begun to prepare M56 constructs with part of the leader peptide sequence deleted. We have found that removal of half of the leader peptide of the HTN polyprotein glycoprotein precursor results in the abolition of G1 and G2 expression, despite the presence of an in-frame ATG 44 nucleotides downstream of our cloning site (data not shown). We attribute this result to a lack of proper initiation of ribosomes at an internal in-frame methionine or to the absolute requirement of the leader peptide for proper translation and/or protein stability during translation.

Numerous reports have shown that vaccinia viruses that express foreign genes offer the prospect of a potential live vaccine (14, 15, 18). The results presented here show that vMP2 (G1 and G2) inoculation raises neutralizing antibodies against HTN in mice, whereas mice infected with v1009 (N) do not seem to have any circulating neutralizing antibodies. We were initially concerned by the possibility that the restricted intracellular localization of G1 and G2 would influence the presentation of the antigen to the murine immune system. Our findings suggest that this is not a problem, and data similar to ours have been reported for other systems. For example, Cheng et al. (3) have shown that when cloned into vaccinia virus, hepatitis B virus large surface protein remained cell associated and yet still elicited reactive antibodies when rabbits were vaccinated. More interestingly, Andrew et al. (1) have shown that vaccinia virus recombinants expressing the SA11 rotavirus VP7 rough-endoplasmic-reticulum-associated glycoprotein raised the same level of neutralizing antibodies as did a vaccinia virus recombinant expressing a secreted VP7 glycoprotein (20).

Since HTN has been identified as the etiologic agent of hemorrhagic fever, the development of a vaccine would be of considerable significance (10). However, studies on protection against HTN are limited owing to the lack of an effective animal model system. As mentioned previously, Korean hemorrhagic fever and its serological relative nephropathia epidemica are believed to be maintained in and transmitted to humans from rodents with no apparent intermediary vector. Lee has demonstrated that large quantities of virus are excreted in the saliva, urine, and feces of *A. agrarius* infected with HTN (10). The infected *A. agrarius* are able to excrete the virus in saliva and feces for 1 month and in urine for 12 months without apparent illness or disease.

Recently, Kim and McKee have reported that intracerebral inoculation of newborn outbred suckling ICR mice with a HTN inoculum of 10 50% lethal doses resulted in a mortality rate of 100% (9). However, this mortality rate declined to 50% if the inoculum was given 7 days after birth. We suspect that the neutralizing antibodies raised from vMP2-infected mice could be used passively in this animal system, and it will be of interest to see whether neutralizing antibodies can protect animals from challenge with live HTN. One limitation of this artificial model system is that mice that die as a result of HTN infection suffer a central nervous system disease with histologic evidence of visceral organ involvement (17), and the clinical and histologic changes characteristic of human hemorrhagic fever with renal syndrome are not observed. Nevertheless, prevention of HTN disease in suckling mice may provide us with an important insight into the natural pathogenicity of the virus to rodents. Use of our vaccinia virus expression system will also offer the opportunity to clone G1 and G2 separately, to define more precisely which glycoprotein is responsible for production of neutralizing antibodies.

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