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Vaccinia Virus Recombinants Expressing either the Measles Virus Fusion or Hemagglutinin Glycoprotein Protect Dogs against Canine Distemper Virus Challenge

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cDNA clones of the genes encoding either the hemagglutinin (HA) or fusion (F) proteins of the Edmonston strain of measles virus (MV) were expressed in vaccinia virus recombinants. Immunofluorescence analysis detected both proteins on the plasma membranes of unfixed cells as well as internally in fixed cells. Immunoprecipitation of metabolically radiolabeled infected-cell extracts by using specific sera demonstrated a 76-kDa HA polypeptide and gene products of 60, 44, and 23 kDa which correspond to a MV F precursor and cleavage products F_0, F_1, and F_2, respectively. Neither recombinant induced cell fusion of Vero cells when inoculated individually, but efficient cell fusion was readily observed upon coinfection of cells with both recombinants. Inoculation of dogs with the vaccinia virus-MV F recombinant (VV-MVF) did not give rise to detectable MV-neutralizing antibody. Inoculation of dogs with the vaccinia virus-MV HA recombinant (VV-MVHA) or coinoculation with both recombinants (VV-MVF and VV-MVHA) induced significant MV-neutralizing titers that were increased following a booster inoculation. Inoculation of dogs with the vaccinia virus recombinants or with MV failed to induce canine distemper virus (CDV)-neutralizing antibodies. Upon challenge with a lethal dose of virulent CDV, signs of infection were observed in dogs inoculated with (VV-MVF). No symptoms of disease were observed in dogs that had been vaccinated with VV-MVHA or with VV-MVHA and VV-MVF and then challenged with CDV. All dogs vaccinated with the recombinant viruses as well as those inoculated with MV or a vaccine strain of CDV survived CDV challenge.

Both canine distemper virus (CDV) and measles virus (MV) are members of the *Morbillivirus* genus of the family *Paramyxoviridae* (33) and contain a nonsegmented single-stranded RNA genome of negative polarity. The characteristics of morbilliviruses have been recently reviewed by Norrby and Oxman (42) and Diallo (15). As reported for other paramyxoviruses (7, 37), two structural proteins are crucial for the induction of a protective immune response. These are the membrane glycoprotein hemagglutinin (HA), which is responsible for hemagglutination and attachment of the virus to the host cell, and the fusion glycoprotein (F), which causes membrane fusion between the virus and the infected cell or between the infected and adjacent cells (23). The order of genes in the MV genome has been deduced by Richardson et al. (51) and Dowling et al. (16). The nucleotide sequences of the MV HA gene and MV F gene have been determined by Alkhatib and Briedis (2) and Richardson et al. (50), respectively.

MV HA and F genes have been expressed in several viral vectors including vaccinia virus (17, 66), fowlpox virus (54, 65), adenovirus (3), and baculovirus (62). In these studies, authentic MV proteins were expressed which were functional in hemagglutination (62), hemolysis (3, 62), or cell fusion (3, 62, 66) assays. When either the HA or F protein is inserted into a vaccinia virus vector, its expression was capable of eliciting a protective immune response in mice against MV encephalitis (17). Similarly, expression of the F protein in a fowlpox virus vector elicited protective immunity against MV encephalitis in mice (65). No protection studies were reported with the other vectors.

CDV is the causative agent of a highly infectious disease of dogs and other carnivores. The properties of the disease have recently been reviewed by Fenner et al. (18). The virus is structurally similar and serologically related to MV (43, 53). Stephenson and ter Meulen (56), Hall et al. (27), and Orvell and Norrby (46) demonstrated that antiserum to MV could immunoprecipitate the HA, P, NP, F, and M polypeptides of both MV and CDV, while antiserum to CDV could precipitate all CDV polypeptides and all MV polypeptides except HA. These data supported previous studies of Orvell and Norrby (45), who described a one-way cross-neutralization between CDV and MV. Similarly, neutralizing antibodies against CDV have been reported in human anti-MV sera (1, 29–31) but neutralizing antibodies against MV have not been found in anti-CDV sera from dogs (13, 31, 52).

In the present study, cDNA copies of the sequences encoding the HA and F proteins of MV (Edmonston strain) were inserted into vaccinia virus to create single recombinants. Both recombinants were shown to express authentic MV proteins. Upon inoculation into beagles, the MV HA recombinant was able to induce MV- but not CDV-neutralizing antibodies. Nevertheless, both MV F and MV HA recombinants induced protective immunity against a lethal challenge with CDV.

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MATERIALS AND METHODS

Cells and virus. The rescuing virus used in the production of the two vaccinia virus recombinants vP455 (VV-MVF) and vP557 (VV-MVHA) was the Copenhagen strain of vaccinia virus from which open reading frame J2R, which encodes the thymidine kinase gene, had been deleted (vP410) (21, 22). All viruses were grown and titers were determined on Vero cell monolayers.

Generation of vaccinia virus recombinant vP557 (VV-MVHA), which contains the MV (Edmonston strain) HA gene. The early/late vaccinia virus H6 promoter (25, 48, 58, 59) was generated synthetically and subsequently inserted into pMP2LVC digested with *Xho*I-*Hin*dIII to yield plasmid pSP131. The plasmid pMP2LVC contains the leftmost 0.4 kbp of the vaccinia virus (Copenhagen strain) *Hin*dIII K (21, 22) region within pUC18. The plasmid contains a multiple cloning region positioned between the sequences corresponding to the K1L and K2L open reading frames of the vaccinia virus genome (21, 22). Annealed oligonucleotides 3P1 (5′-GGGAAGATGGAACCAATCGCAGATAG-3′) and 3P2 (5′-AATTCTATCTGCGATTGGGGTTCCATCTTCCC-3′) which contain the extreme 3′ sequences of the HA gene and a sticky *Eco*RI end a 1.8-kbp *Xho*I-*Sma*I fragment from pMH22 which contains the remainder of the HA gene were ligated with *Xho*I-*Eco*RI-digested pSP131. The resultant plasmid was designated pSPMHA11. Plasmid pMH22 was derived from a full-length cDNA clone of the MV HA gene by creating a *Xho*I site at the ATG initiation codon (2).

A 1.9-kbp *Hin*dIII-*Eco*RI fragment from pSPMHA11, which contains the MV HA gene, was isolated and blunt ended with the Klenow fragment of the *Escherichia coli* DNA polymerase in the presence of 2 mM deoxynucleoside triphosphates. The isolated fragment was inserted into pMP409DVC (24) which had been digested with *Bgl*II and blunt ended by treatment with mung bean nuclease. Insertion into this vector yielded plasmid pSPMHA41. The *Xho*I site between the H6 promoter and the initiation codon of the HA gene was removed by oligonucleotide-directed double-strand break mutagenesis (36) by using oligonucleotide HAXHOD (5′-ATATCCGTTAAGTTTGTATCGTAATGT CACCACAACGAGACCGGAT-3′), thus generating plasmid pSPM2LHA. Insertion plasmid pSPM2LHA was used in in vivo recombination, with vaccinia virus vP410 as the rescue virus, to generate recombinant vP557 (VV-MVHA). This vaccinia virus recombinant contains the MV HA gene in the M2L locus of the genome. Generation of plasmid pSPM2LHA is described in Fig. 1.

Generation of vaccinia virus recombinant vP455 (VV-MVF) which contains the MV F gene. Annealed oligonucleotides 3PA (5′-CCTAAAGCCTGATCTTACGGGAACATCAAAATCC TATGTAAGGTCGCTCTGATTTTTATCGGCCGA-3′) and 3PB (5′-AGCTTCGGCCGATAAAAATCAGAGCGACCTT ACATAGGATTTTGATGTTCCCGTAAGATCAGGCTTT AGG-3′) which contain the 3′ end of the MV F gene, a vaccinia virus early transcription termination signal (68), and *Eag*I and *Hin*dIII ends were ligated to a 1-kbp *Sal*I-*Hae*III fragment from pCRF2 (which contains a full-length cDNA of the MV F gene) and pUC8 digested with *Sal*I and *Hin*dIII. The resulting plasmid, pMF3PR14, contains the 3′ (1 kbp) end of the MV F gene. Annealed oligonucleotides 5PA (5′-GGGA TGGGTCTCAAGGTGAACGTCTCTGCCATATTC-3′) and 5PB (5′-ATGGCAGAGACGTTCACCTTGAGACCCATCC C-3′), which contain a 5′ *Sma*I site and a 3′ *Bst*XI site, were ligated to an 820-bp *Bst*XI-*Sal*I fragment from pCRF2 and pUC8 which had been digested with *Sma*I and *Sal*I. The resultant plasmid, pSPMF5P16, contains the 5′ portion of the MV F gene. The 850-bp *Sma*I-*Sal*I fragment from pSPMF5P16 and the 1-kbp *Sal*I-*Eag*I fragment from pMF3PR14 were ligated into pTP15 (25) which had been digested with *Sma*I and *Eag*I. The resultant plasmid containing the MV F gene juxtaposed 3′ to the H6 promoter within the HA insertion plasmid was designated pSPHMF7. Oligonucleotide-directed mutagenesis (34) was performed on pSPHMF7. Initially, the *Bgl*II site at the 5′ end of the H6 promoter was removed by using oligonucleotide SPBGLD (5′-AATAAA TCACTTTTTATACTAATTCTTTATTCTATACTTAAAA AGT-3′) and following the Mandecki procedure (36). A subsequent mutagenesis reaction was performed to create a precise ATG-ATG linkage of the H6 promoter with the MV F gene. This was performed using the Mandecki procedure with the oligonucleotide SPMAD (5′-TATCCGTTAAGTTT GTATGGTAATGGGTCTCAAGGTGAACGTCT-3′). The resultant plasmid was designated pSPMFVC. This plasmid was used in in vivo recombination, with vaccinia virus vP410 as the rescue virus, to generate vP455 (VV-MVF). Generation of plasmid pSPMFVC is described in Fig. 2.

Cell fusion experiments. Vero cell monolayers in 60-mm-diameter dishes were inoculated at a multiplicity of infection of 1 PFU per cell with parental or recombinant viruses. After 1 h of absorption at 37°C the inoculum was removed, the overlay medium was replaced, and the dishes were incubated overnight at 37°C. At 20 h postinfection, plates were examined with a Nikon Diaphot inverted microscope equipped with phase-contrast optics and, using a 20× objective lens, were photographed with Kodak Technical Pan film ASA 25.

Immunoprecipitation experiments. Immunoprecipitation experiments were performed essentially as described by Taylor et al. (57). The fusion protein was specifically precipitated by using a rabbit antiserum directed against a carboxy terminal fusion peptide (62). The HA protein was specifically precipitated by using a monospecific polyclonal anti-HA serum.

Immunization of dogs. Fourteen 10-week-old specific-pathogen-free beagles from the James A. Baker Institute colony were studied. Blood samples were collected at the initiation of the experiment and repeatedly thereafter. Four groups with two dogs in each group were immunized with two injections three weeks apart. The first group received vaccinia virus only. The second group received vaccinia virus with an insert for the F protein of MV (vP455). The third group received vaccinia virus with an insert for the HA antigen of MV (vP557), and the fourth group received a combination of the two vaccinia virus recombinants. Each dog was inoculated with approximately 4×10^8 PFU of vaccinia virus in 1-ml amounts (0.6 ml subcutaneously and 0.4 ml intramuscularly). Two control dogs received 10^5 50% tissue culture infectious doses ($TCID_{50}$) of the attenuated Edmonston strain of MV intramuscularly (1-ml amount) and two control dogs received 10^4 $TCID_{50}$ of the attenuated Rockborn strain of CDV subcutaneously 2 weeks before challenge with virulent CDV. Two control dogs remained uninoculated. All dogs were challenged by intranasal inoculation of 1 ml of tissue culture fluid containing 10^4 $TCID_{50}$ of the Snyder Hill strain of virulent CDV 2 weeks after the last inoculation. The clinical reactions of the dogs were monitored by daily observation and by recording body temperature and weight gains or losses. Circulating blood lymphocytes were counted before challenge and 3, 5, 7, and 10 days postchallenge. Virus isolation from buffy coat cells by cocultivation with dog lung macrophages (5) was attempted 3, 5,

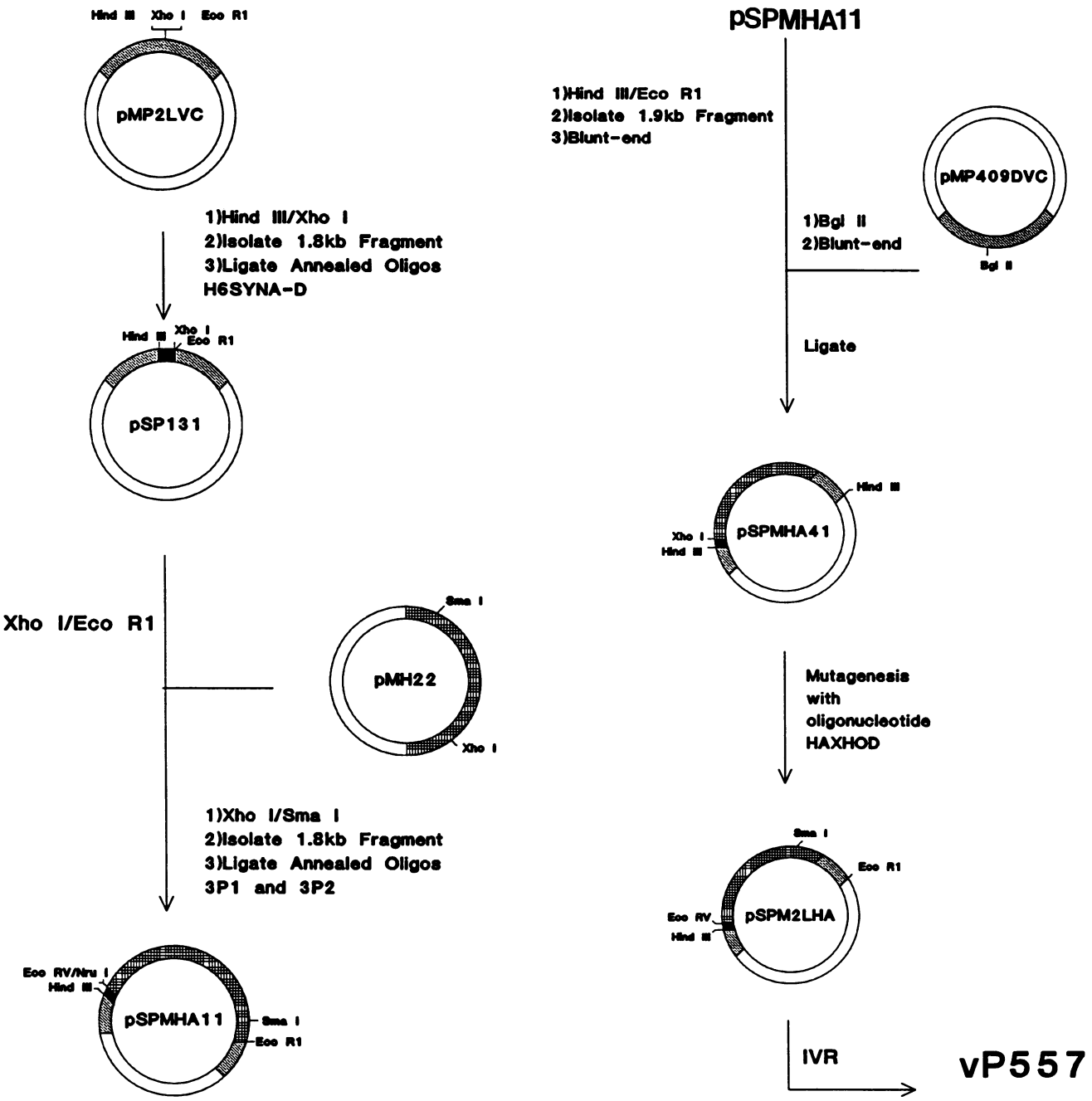

FIG. 1. Generation of a vaccinia virus recombinant which expresses MV HA. Insertion plasmid pSPM2LHA was constructed as described in Materials and Methods and is schematically represented here. This plasmid was used in in vivo recombination experiments, with a *tk* deletion mutant of the Copenhagen strain of vaccinia virus (vP410) as the rescue virus, to generate vP557. Plasmid sequences are represented by open regions, vaccinia virus sequences are represented by diagonal lines, MV HA is represented by hatched boxes, and the vaccinia virus H6 promoter is represented by the darkened region.

7, and 10 days postchallenge. Blood samples for serological tests were collected before vaccination, at weekly intervals until the time of challenge, and 7, 10, and 20 days postchallenge.

Serological tests. The technique for virus neutralizing antibody testing was previously described in detail (4). Testing for CDV-neutralizing antibody titers was performed on Vero cells with the adapted Onderstepoort strain of CDV. Testing for MV-neutralizing antibody titers involved the adapted Edmonston strain of MV, also on Vero cells.

RESULTS

Vaccinia virus recombinants expressing the MV F or HA genes were constructed. These reagents allowed us to address the immunogenic potential of these MV antigens and

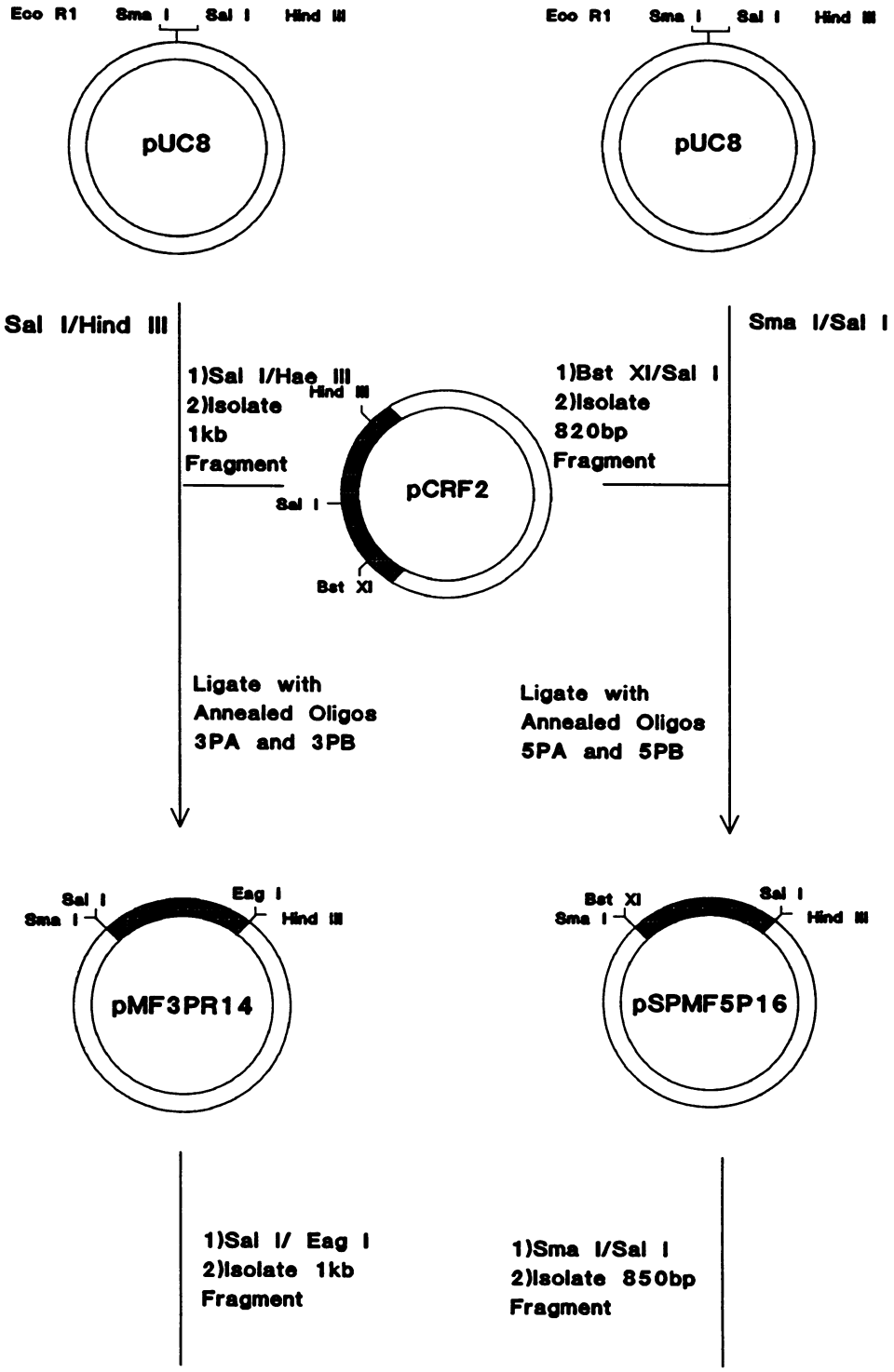

FIG. 2. Generation of a vaccinia virus recombinant which expresses the MV F protein. Insertion plasmid pSPMFVC was constructed as described in Materials and Methods and is schematically represented here. This plasmid was used in in vivo recombination experiments, with a *tk* deletion mutant of the Copenhagen strain of vaccinia virus (vP410) as the rescue virus, to generate vP455. Plasmid sequences are represented by open segments, vaccinia virus sequences are represented by diagonal lines, MV F is represented by hatched boxes, and the vaccinia virus H6 promoter is represented by the darkened regions.

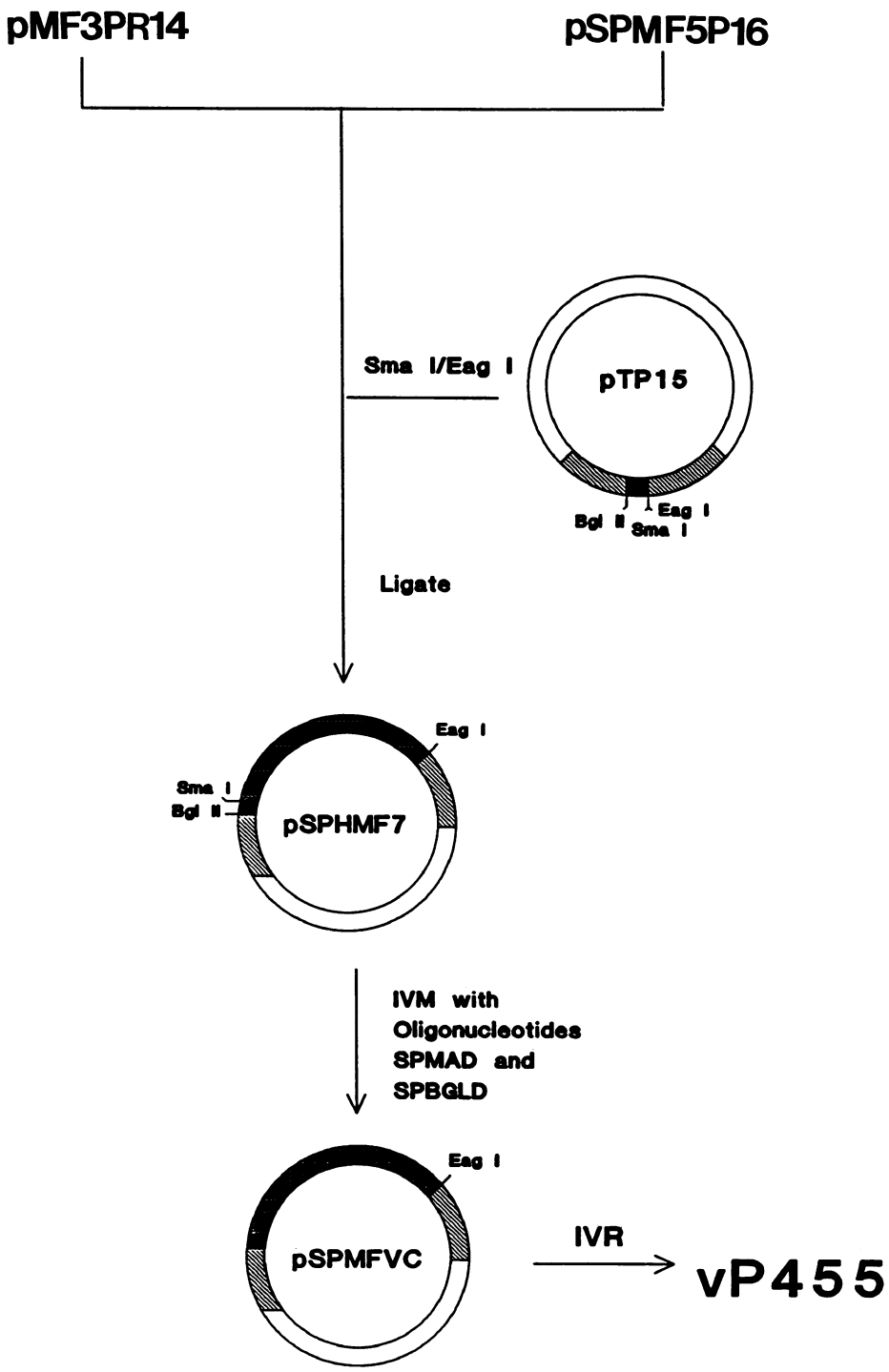

FIG. 2—*Continued.*

furthermore, to decipher in a dog model the cross-relationships between two members of the *Morbillivirus* genus MV and CDV, at both the serological level and the level of cross-protection against a virulent CDV challenge.

Construction of vaccinia virus recombinants which express extrinsic MV F or HA antigens. Insertion of the sequences that encode the MV F and HA proteins resulted in the development of recombinants vP455 (expressing MV F) and vP557 (expressing MV HA) (Fig. 1 and 2).

Immunoprecipitation analysis of MV F and HA products expressed by vaccinia virus recombinants. In order to determine that both recombinants expressed authentic proteins, immunoprecipitation experiments were performed. Briefly, Vero cell monolayers were infected at 10 PFU per cell with

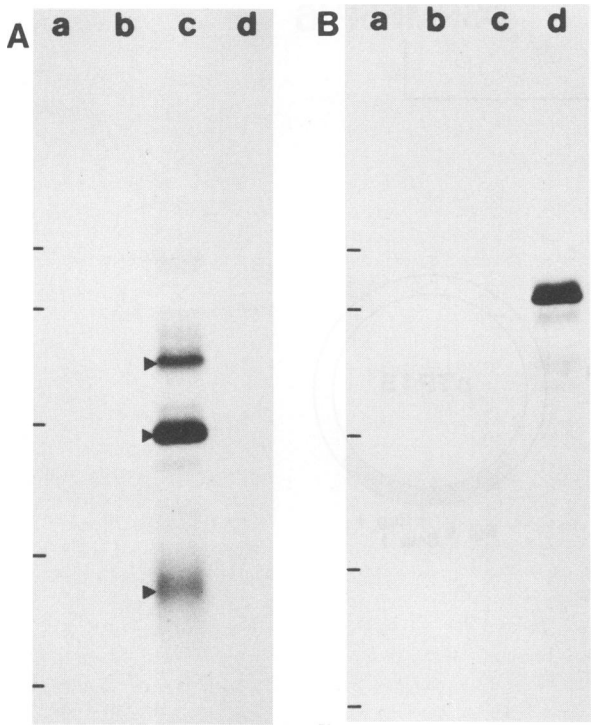

FIG. 3. Immunoprecipitation of F and HA proteins from recombinant vaccinia virus-infected Vero cells. Cells were infected at 10 PFU per cell with parental vP410 or with recombinants vP455 or vP557. [^{35}S]methionine was added at 1 h postinfection, and the incorporation was allowed to proceed until 8 h postinfection, when cultures were harvested and cells were lysed with a nonionic detergent. Immunoprecipitations were performed by using a monospecific rabbit anti-F serum (A) or a specific polyclonal rabbit anti-HA serum (B). Lanes a, uninfected Vero cells; lanes b, parental virus; lanes c, vP455; and lanes d, vP557. Marks to the left of lanes a indicate migration distances for marker proteins with molecular masses (from the top of the gel) of 200, 97.4, 68, 43, 29, 18.4, and 14.3 kDa.

either parental or recombinant viruses in the presence of [^{35}S]methionine. Specific proteins were immunoprecipitated from the infected-cell lysate as described in Materials and Methods. Figure 3A illustrates immunoprecipitation with an F protein-specific serum. No radiolabeled products were detected in uninfected Vero cells (Fig. 3A, lane a), in wild-type-virus-infected Vero cells (Fig. 3A, lane b), or in cells infected with the HA recombinant vP557 (Fig. 3A, lane d). Figure 3A, lane c, represents immunoprecipitation of proteins from cells infected with the F recombinant vP455. The F precursor F_0, which has a molecular mass of approximately 60 kDa, and the cleavage products F_1 and F_2, which have molecular masses of approximately 44 and 23 kDa, respectively, are clearly visible. Figure 3B depicts immunoprecipitation of the MV HA protein, which has a molecular mass of approximately 76 kDa. Authentic glycosylation of the protein is assumed on the basis of mobility. No products were detected in uninfected Vero cells (Fig. 3B, lane a), in wild-type-virus-infected cells (Fig. 3B, lane b), or in Vero cells infected with the VV-MVF recombinant vP455 (Fig. 3B, lane c). In addition, immunofluorescence studies indicated that both proteins were present on the infected-cell surface (data not shown).

Analysis of syncytium formation on Vero cells infected with VV-MVF and VV-MVHA recombinants. A characteristic of MV cytopathology is the formation of syncytia by fusion of infected cells with surrounding infected or uninfected cells, which is followed by migration of the nuclei toward the center of the syncytium (39). This has been shown to be an important method of viral spread, which for paramyxoviruses can occur in the presence of viral HA-specific virus neutralizing antibody (37). In order to determine that the MV proteins expressed in vaccinia virus were functionally active, Vero cell monolayers were inoculated with recombinants at 1 PFU per cell and the cytopathic effects were observed microscopically at 18 h postinfection. The results of the experiment are shown in Fig. 4. No cell fusing activity was evident in Vero cells inoculated with the wild-type parental vaccinia virus (Fig. 4B), with the MV F recombinant vP455 (Fig. 4C), or with the MV HA recombinant vP557 (Fig. 4D). However, when vP455 and vP557 were coinoculated, efficient cell-fusing activity was observed (Fig. 4E). This result has recently been confirmed by Wild et al. (66), who determined that syncytium formation in a variety of cell lines that had been infected with MV-vaccinia virus recombinants required expression of both the F and HA genes. This result, however, is in contrast to that obtained by Alkhatib et al. (3) who observed cell fusion in 293 cells infected with high multiplicities of an adenovirus recombinant expressing only the MV F protein. Similarly, Vialard et al. (62) observed cell fusion in insect cells infected with a baculovirus recombinant expressing the MV F protein, but only when they were incubated at pH 5.8. In neither case was the fusion activity enhanced by coinfection with the appropriate recombinant expressing the MV HA protein. While hemagglutination was evident between African green monkey erythrocytes and insect cells infected with a baculovirus-MV HA recombinant, no aggregation could be demonstrated between recombinant-infected insect cells (62). This may indicate that no receptor for MV HA exists on

TABLE 1. MV-neutralizing antibody induced in dogs inoculated with vaccinia virus recombinants expressing MV F or HA proteins

Virus[a]	Dog no.[b]	Antibody titer at day prechallenge:				
		−35[c]	−28	−14[d]	−7	0[e]
VV	4/1	<1	<1	<1	<1	<1
	4/2	<1	<1	<1	<1	<1
VV-MVF	4/3	<1	<1	<1	<1	<1
	4/4	<1	<1	<1	<1	<1
VV-MVHA	4/5	<1	2.2[f]	2.9	3.4	3.4
	4/6	<1	2.7	2.9	3.9	3.4
VV-MVF+HA	4/7	<1	2.7	3.2	3.4	3.4
	4/8	<1	2.5	2.9	3.6	2.9
MV	4/14			<1[a]		2.9
	4/15			<1		3.2

[a] VV, vaccinia virus.
[b] Dogs 4/1 to 4/8 received 4×10^8 PFU of vaccinia virus or recombinants. Dogs 4/14 and 4/15 received 10^5 $TCID_{50}$ of the Edmonston strain of MV.
[c] First immunization.
[d] Second immunization.
[e] Time of challenge.
[f] Titer expressed as $\log_{10}$ of the last antibody dilution showing complete neutralization of infectivity in a microtiter neutralization test, as described by Appel and Robson (4).

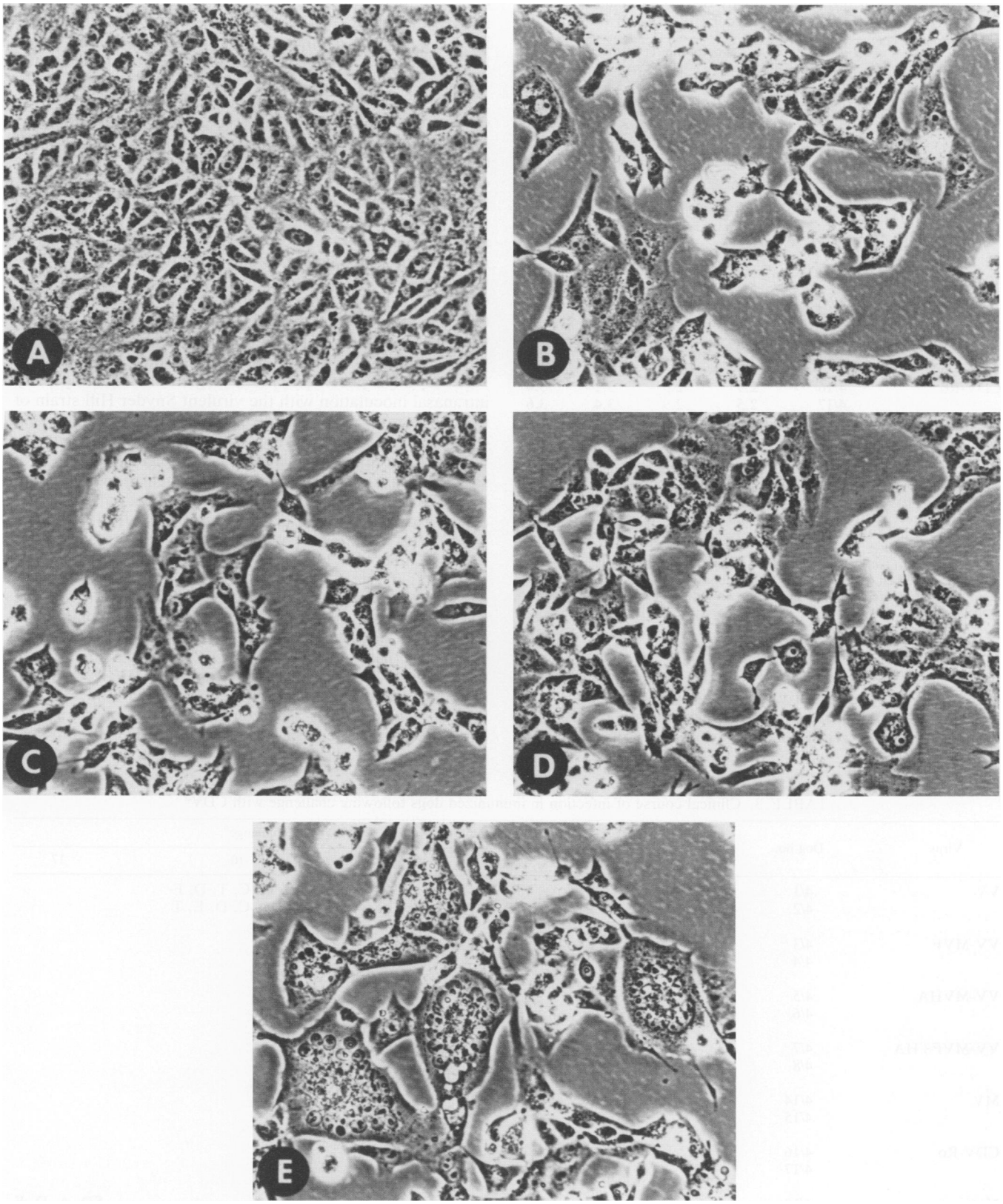

FIG. 4. Analysis of fusion activity of MV glycoproteins expressed by vaccinia virus. Vero cell monolayers were inoculated at 1 PFU per cell and examined microscopically at 18 h postinfection. (A) uninfected cells, (B) parentally infected cells, (C) vP455-infected cells, (D) vP557-infected cells, (E) vP455 and vP557 mixed infection.

TABLE 2. CDV-neutralizing antibody titers after challenge with 10^4 $TCID_{50}$ of the virulent Snyder Hill strain of CDV

Virus	Dog no.[a]	Antibody titer at day post-CDV challenge:			
		0[b]	7	10	20
VV	4/1	<1	<1	<1	—
	4/2	<1	<1	<1	—[c]
VV-MVF	4/3	<1	<1	2.2	2.5
	4/4	<1	<1	2.2	2.5
VV-MVHA	4/5	<1	<1	2.9	2.7
	4/6	<1	<1	<1	<1
VV-MVF+HA	4/7	<1	<1	3.4	3.6
	4/8	<1	<1	3.6	3.4
MV	4/14	<1	<1	2.5	2.2
	4/15	<1	<1	2.9	3.2
CDV-Ro	4/16	2.9[d]	2.5	3.2	3.4
	4/17	2.5	2.9	3.4	3.6
None	4/18	<1	<1	<1	—
	4/19	<1	<1	—	—

[a] Dogs 4/1 and 4/8 received 4×10^8 PFU of vaccinia virus recombinants, dogs 4/14 and 4/15 received 10^5 $TCID_{50}$ of the Edmonston strain of MV, dogs 4/16 and 4/17 received 10^4 $TCID_{50}$ of the attenuated Rockborn strain of CDV, and dogs 4/18 and 4/19 were not immunized.

[b] Day of challenge.

[c] —, dogs were euthanized when pronounced dehydration was evident.

[d] Titer expressed as $\log_{10}$ of the last antibody dilution showing complete neutralization of infectivity in a microtiter neutralization test as described by Appel and Robson (4).

insect cells, which would explain the lack of synergism between the HA and F proteins in the fusion process.

Serological and protective immune responses induced by vaccinia virus-MV recombinants in dogs. In order to determine whether expression of the MV proteins in dogs inoculated with the vaccinia virus recombinants was sufficient to induce a protective immune response against CDV challenge, beagles were inoculated as described in Materials and Methods.

Dogs immunized with either the wild-type parental vaccinia virus or the vaccinia virus recombinant expressing the MV F protein did not develop neutralizing antibodies to MV (Table 1), even after a booster inoculation. Dogs that were either immunized with vP557, which expressed the MV HA protein, or coinoculated with both recombinants vP455 and vP557 did develop high levels of MV-neutralizing antibodies after 1 week and a single inoculation (Table 1). Levels of neutralizing antibody were equivalent to those induced by inoculation with the attenuated Edmonston strain of MV (Table 1). A booster inoculation in dogs 4/5, 4/6, 4/7, and 4/8 (Table 1) resulted in increased levels of neutralizing antibodies. Significantly, neither MV nor the recombinant vaccinia viruses induced CDV-neutralizing antibodies (Table 2).

At 35 days postvaccination, dogs were challenged by intranasal inoculation with the virulent Snyder Hill strain of CDV. The results of the challenge are shown in Tables 3 and 4. Nonimmunized control dogs and dogs vaccinated with the wild-type parental vaccinia virus developed clinical signs of severe disease and were euthanized when pronounced dehydration was evident. Both dogs immunized with the vaccinia virus recombinant expressing MV F (vP455) showed some signs of infection with CDV including weight loss, lethargy, anorexia, elevated body temperature, and lymphopenia. These symptoms were milder and of shorter duration than those seen in control dogs. Infection with the CDV challenge virus was confirmed by virus isolation on days 5 and 7 postchallenge (Table 4). Nonetheless, both dogs survived lethal challenge with CDV. Dogs inoculated with the vaccinia virus recombinant expressing MV HA (vP557) or coinoculated with both the MV F (vP455) and MV HA (vP557) vaccinia virus recombinants showed no significant clinical signs of infection and survived challenge, although

TABLE 3. Clinical course of infection in immunized dogs following challenge with CDV[a]

Virus	Dog no.	Symptom(s) at day post-CDV-SH challenge[b]:				
		4	6	8	10	17
VV	4/1	L, A, T	L, A	SD, A, C	SD, A, C, T, D, E	
	4/2	L, A, T	L, A	SD, A, C, T	SD, A, C, D, E, T	
VV-MVF	4/3	L, A, T	L, A	L, A, T	T	
	4/4	L, A, T	L, A, T			
VV-MVHA	4/5		T			
	4/6					
VV-MVF+HA	4/7					
	4/8		T			
MV	4/14					
	4/15					
CDV-Ro	4/16					
	4/17					
None	4/18		L, A, T			SD, A, D, E
	4/19	L, A, T	L, A, T	SD, A, C	SD, A, C, D, E	

[a] Animals were challenged by intranasal inoculation of 10^4 $TCID_{50}$ of the Snyder Hill strain of virulent CDV (CDV-SH) 14 days after the final immunization.

[b] L, lethargy; A, anorexia; SD, severe depression; T, elevation of body temperature above 39.5°C; C, conjunctivitis; D, dehydration; E, euthanized.

TABLE 4. Laboratory confirmation of infection of immunized dogs following challenge with CDV[a]

Virus	Dog no.	Wt loss (kg) and method of confirmation of infection at day post-CDV-SH challenge[b]:				
		3	5	7	10	13
VV	4/1	0.1, V	V	0.9, Ly, V	1.5, Ly	
	4/2	0.1, Ly, V	Ly, V	0.3, Ly, V	1.1, Ly	
VV-MVF	4/3		Ly, V	0.2, Ly, V	0.4	
	4/4		V	0.2, Ly, V	1.1	
VV-MVHA	4/5			V	Ly	
	4/6					
VV-MVHA+F	4/7			Ly, V		
	4/8			0.3, V		
MV	4/14			Ly		
	4/15		Ly	0.3		
CDV-Ro	4/16					
	4/17					
None	4/18			0.1	0.3, V	Ly, V
	4/19	0.2, Ly, V	V	0.5, Ly, V	1.4, Ly, V	

[a] Animals were challenged by intranasal inoculation of 10^4 $TCID_{50}$ of the virulent Snyder Hill strain of CDV (CDV-SH) 14 days after the final inoculation.
[b] V, virus isolated by cocultivation of buffy coat cells with dog lung macrophages as described in Appel and Jones (5); Ly, lymphopenia evidenced by a total lymphocyte count below 2×10^3 lymphocytes per mm^3.

virus was isolated from three of the four dogs on day 7 postchallenge. Dogs inoculated with either the attenuated Edmonston strain of MV or the attenuated Rockborn strain of CDV also survived challenge with no signs of disease. No CDV challenge virus was recovered from the CDV (Rockborn strain)- or MV-immunized dogs when buffy coat cells were cocultivated with dog lung macrophages.

DISCUSSION

This study describes the generation of vaccinia virus recombinants which express the MV F and MV HA glycoproteins. Both proteins become inserted into the plasma membrane of cells infected with the recombinants and are expressed in an authentic fashion. MV HA is evident in VV-MVHA (vP557)-infected cells as a 76-kDa glycoprotein, while MV F in VV-MVF (vP455)-infected cells is present as a 60-kDa precursor protein (F_0) that is proteolytically cleaved into the 44-kDa (F_1) and 23-kDa (F_2) mature forms.

Contrary to previous publications which indicate that expression of the F protein alone is sufficient to induce syncytium formation (3, 62), no fusogenic activity was observed in Vero cells inoculated with either the VV-MVF or VV-MVHA recombinants. These results could be explained by differences in the pH of the overlay medium or in the cell type. Giraudon et al. (20) found that persistently infected BGM cells (a line of African green monkey kidney cells) did not exhibit synctytium formation, even though both glycoproteins were present on the infected-cell surface. A potential controlling factor may also be the level of expression, since Norrby et al. (39) suggested that lack of fusion activity in persistently infected cells may be due to lower levels of expression of the F protein. Nonetheless, when the VV-MVF and VV-MVHA recombinants were coinoculated onto Vero cells, efficient cell fusing activity was evident (Fig. 4). The result has recently been confirmed by Wild et al. (66), who determined that syncytium formation required expression of both HA and F genes in a vaccinia virus vector.

In order to evaluate the efficacy of the VV-MVF and VV-MVHA recombinants, the close immunological relationship between MV and CDV was utilized. Both MV and CDV are members of the *Morbillivirus* genus. It has been previously demonstrated that protection against CDV in dogs can be induced by vaccination with MV (19, 38, 63). Upon inoculation of dogs, no anti-MV neutralizing antibody was detectable in dogs inoculated with VV-MVF (vP455) (Table 1). In a subsequent study, however, one beagle inoculated with VV-MVF (vP455) did develop serum-neutralizing antibody after two inoculations (59a). In addition, guinea pigs and rabbits inoculated subcutaneously with VV-MVF (vP455) all developed low levels of MV-neutralizing antibody (unpublished results). It has been documented that F-specific antibodies have low neutralizing activity in in vitro tests (41), although Malvoisin and Wild (35) have recently isolated anti-F monoclonal antibodies which effectively neutralize virus infectivity. Expression in vaccinia virus vectors of the F protein of other paramyxoviruses, respiratory syncytial virus (64), parainfluenza virus type 3 (55), and rinderpest virus (9, 67) has resulted in the development of serum-neutralizing antibodies in animals inoculated with the recombinant. Although dogs vaccinated with VV-MVF (vP455) exhibited some clinical symptoms, they were protected against the lethal live CDV challenge (Table 3). Dogs that were either inoculated with VV-MVHA (vP557) or coinoculated with VV-MVF and VV-MVHA presented minimal clinical manifestations, developed levels of neutralizing antibody equivalent to that induced by live MV vaccination, and were fully protected against CDV-induced death (Tables 3 and 4).

Although these results would indicate that expression of HA alone is sufficient for induction of protective immunity, Norrby et al. (40, 41) suggested that vaccine failures evident in killed-MV vaccines were due to inactivation of the fusion component. In addition, protection against both CDV chal-

lenge and MV challenge has been achieved using either adjuvented isolated fusion proteins (44) or fusion proteins presented in an immune-stimulating complex (14, 61). In the latter study, the F-immune-stimulating complex preparation failed to elicit virus-neutralizing antibodies in monkeys but did activate MV-specific T cells in mice and protect them against fatal MV encephalopathy (14). This raises the question of the importance of cell-mediated immunity in protection against MV infection. In addition, while antibodies to the F protein may not have been able to neutralize the challenge virus and inhibit infection, they may have played a role in limiting the spread of infection and preventing a fatal outcome.

CDV is a highly infectious disease of dogs which occurs worldwide. The mortality rate is high (between 30 and 80%), and surviving dogs often have permanent central nervous system damage (18). Immunization by using a live attenuated vaccine at 8 weeks of age and again at 12 to 16 weeks of age is recommended for control of CDV. Neutralizing antibodies are transferred to offspring in the colostrum. Although immunity after virulent CDV infection is lifelong, some dogs lose immunity 1 or 2 years after vaccination, and annual revaccination is usually recommended.

One problem with the current policy of continual revaccination is the transfer of neutralizing antibodies from CDV-immune mothers to offspring in the colostrum. It is difficult to ascertain when these antibody levels will wane to such a level that pups can be vaccinated. This leaves a period during which pups may be susceptible to CDV infection. Use of a recombinant vaccine expressing the MV glycoproteins may provide a means to overcome the inhibitory effects of the maternal antibodies and allow vaccination of young pups. In fact, it has been demonstrated that CDV-specific antibodies in pups that suckled CDV-immune mothers did not prevent the development of MV-specific antibodies in the pups when they were inoculated with a MV vaccine (8).

Live attenuated vaccines against CDV have been used very successfully for a number of years, although some limitations of their use have been documented (60). These vaccine-associated problems are linked to the ability of the live vaccine strains to replicate within the recipient animal and are most notable when the CDV vaccine strain is coinoculated in dogs with adenovirus types 1 and 2, resulting in immunosuppression, thrombocytopenia, and encephalitis (10, 28, 49). The modified live CDV vaccines have also been shown to induce distemper in other animal species including foxes, kinkajous, ferrets, and pandas (11, 12, 26, 32), although there are no data to indicate that a MV recombinant would be protective in these species. The use of a recombinant CDV-vaccinia virus candidate would eliminate the continued introduction of modified live CDV into the environment. In addition, it has been demonstrated that vaccinia virus recombinants are safe to use in dogs and do not induce side effects (this study and reference 6).

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