Evidence that Measles Virus Hemagglutinin Initiates Modulation of Leukocyte Function-Associated Antigen 1 Expression

ATTIBELE R. NAGENDRA,¹ C. WAYNE SMITH,² AND PHILIP R. WYDE^{1*}

Department of Microbiology and Immunology¹ and Laboratory of Leukocyte Biology,
Department of Pediatrics,² Baylor College of Medicine, Houston, Texas 77030

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Measles virus (MV), human immunodeficiency virus, Epstein-Barr virus, and other leukotropic viruses can modulate the expression of leukocyte function antigen 1 (LFA-1) on the surface of infected and nearby leukocytes. This ability to induce changes in LFA-1 expression may play an important role in the pathogenesis of these viruses. However, the mechanism(s) involved in virus-mediated regulation of LFA-1 is unknown. Evidence is presented in this report that it is the MV hemagglutinin (H) protein that initiates up-regulation of LFA-1 expression in leukocyte cultures infected with this virus. Indeed, comparison of the abilities of different MV strains to modulate LFA-1 expression, examination of published nucleotide sequences for the H proteins of different vaccine strains, and competitive inhibition assays using oligopeptides homologous or heterologous to a region of the H protein gene encompassing amino acid 116 (from the amino terminus) all suggest that it is this portion of the H protein that is responsible for MV-induced alteration of LFA-1. These comparisons also support the hypothesis that there is a relationship between the abilities of different MV strains to alter LFA-1 expression and their pathogenic potentials.

Measles virus (MV) belongs to the virus family *Paramyxoviridae*. It is a negative-stranded RNA virus bounded by a coat composed predominantly of two glycoproteins, designated hemagglutinin (H) and fusion (F), and a lipid envelope derived from the cytoplasmic membrane of the host cell (25, 29). The H protein contains sites involved in the attachment of MV to host cell receptors and also has a role in the fusion of MV-infected cells (28). The F protein is critical for virus entry into host cells and for the fusion of MV-infected cells. The latter results in the formation of giant cells, a hallmark of measles infection (8).

MV kills over 1 million children annually, primarily as a consequence of pneumonia (3, 5). However, although the primary site of MV infection is the lung, this virus frequently disseminates throughout the body and can cause an array of debilitating disorders, including those involving the liver, pancreas, immune system, and central nervous system (14, 18, 21, 23–25, 33). MV commonly infects leukocytes, and it is infected leukocytes that probably provide the major means by which MV translocates from the respiratory tract (13, 21). MV-leukocyte interactions probably also play a critical role in two other prominent aspects of MV pathogenesis, marked immunosuppression of the host and the frequent occurrence of secondary infections (7, 21).

Leukocyte function-associated antigen 1 (LFA-1) is an integrin, a member of a family of molecules which are differentially expressed on the surface of leukocytes (11, 26). It is a heterodimeric transmembrane protein composed of an alpha chain (CD11a) and a beta chain (CD18). Proper expression of LFA-1 has been reported to be necessary for the induction of many types of host immune responses (19), for the adherence of leukocytes to vascular endothelial and epithelial cells (11),

and for translocation of leukocytes into different tissues (11, 27). Recently it has been reported that several leukotropic viral pathogens of humans (i.e., MV [4, 36], human immunodeficiency virus [12, 31], and Epstein-Barr virus [26, 30]) are able to alter LFA-1 expression following infection of leukocytes. These findings have led to the speculation that the capacity of these viruses to regulate LFA-1 expression is directly related to their ability to disseminate in and to immunosuppress the host following infection (4, 29, 31). However, virtually nothing is known about the mechanism(s) involved in virus-induced modulation of LFA-1 expression.

In studies of MV-induced modulation of LFA-1 (4, 36), we have shown that (i) certain MV strains can modulate expression of LFA-1 on the cytoplasmic membranes of MV-infected leukocytes; (ii) wild-type (WT) MV strains are better modulators of LFA-1 expression than more-attenuated live virus vaccine strains; (iii) MV Edmonston-Zagreb (E-Z), a live virus vaccine strain that may have some pathogenicity (2) and immunosuppressive properties (17), is a better modulator of LFA-1 expression than MV Enders-Moraten (E-M), a vaccine strain that is standardly used in the United States and for which there is no associated pathogenicity; and (iv) following infection of cultured leukocytes by the WT or E-Z strain of MV, there occurs concomitantly an alteration of LFA-1 expression and aggregation of the leukocytes which appears to foster dissemination of virus throughout the infected culture.

In this study, we use the MV-leukocyte model to begin to investigate the mechanism(s) involved in virus-induced alteration of LFA-1 expression on leukocytes. We present evidence indicating that it is the MV H protein, and particularly the region encompassing amino acid site 116 (starting from the amino terminus) of this protein, that is responsible for triggering the activation of LFA-1 which is seen following infection of these cells with MV. Moreover, comparison of the nucleotide sequences in the H protein genes of different MV strains supports the earlier supposition that there is a relationship be-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-5255. Fax: (713) 798-6802. Electronic mail address: pwyde@bcm.tmc.edu.

TABLE 1. Amino acid compositions and corresponding regions of the MV H protein genes of the synthetic oligopeptides used

Oligopeptide	Composition ^a	Amino acid positions	Homologous to the H protein of MV:
EZOP	NH ₂ -WRFTDLVK <u>F</u> ISDKIKFLNPD-COOH	108-127	E-Z
EMOP	NH ₂ -WRFTDLVK <u>L</u> ISDKIKFLNPD-COOH	108-127	E-M
MV03	NH ₂ -LHRAAIYTAEIHKSLSTNLD-COOH	60-79	E-Z
MV04	NH ₂ -LAVSKGNCSGPTTIRGQFSN-COOH	181-200	E-Z

^a Underlined nucleotides indicate the lone differences in the nucleotide sequences of EZOP and EMOP.

tween the abilities of different MV strains to alter LFA-1 expression and their pathogenic potentials (36).

MATERIALS AND METHODS

Tissue culture. Vero (African green monkey kidney) cells obtained from the American Type Culture Collection (catalog no. CCL 23) were used to grow and assay for MV, vaccinia virus (VV), and recombinant VV expressing either MV H (rVV-H) or MV F (rVV-F) glycoprotein. This cell line was propagated in monolayer culture in Earle's minimum essential medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium bicarbonate (0.2%), and L-glutamine (2 mM/ml). Flasks containing these cells were serially passaged whenever they became confluent.

U937 monocytoid cells were used to study MV- and MV H protein-induced effects on LFA-1 expression, since earlier studies (4) had shown no differences in the ability of different MV strains to alter LFA-1 expression on these cells and monocytic cells obtained from freshly drawn human blood, using Ficoll-Hypaque (6). The U937 cells were obtained from the American Type Culture Collection (catalog no. CRL1593) and propagated in suspension in RPMI 1640 medium (catalog no. 12702Y; Whittaker Bioproducts, Walkersville, Md.) supplemented similarly as the minimal essential medium (10% FCS-RPMI). Only cultures with ≥90% viability (as determined by trypan blue exclusion) (22) were used in experiments.

Viruses. VV, rVV-H, and rVV-F were all obtained from Enzo Paoletti, Virogenetics Inc., Troy, N.Y. The construction and characterization of these recombinant viruses have been described in detail elsewhere (9). It should be noted that the MV gene sequences of both rVV-H and rVV-F were derived from the Edmonston strain of MV.

WT MV was acquired from Gail Demmler, Department of Pediatrics, Baylor College of Medicine, Houston, Tex. Live attenuated MV E-M (vaccine strain) was obtained from the Acute Respiratory Disease Center (Baylor College of Medicine). This vaccine was produced by Merck Sharp & Dohme in chick cells and was sold under the trade name Attenuvax. MV E-Z (vaccine strain) was obtained from Lauri Markowitz, Division of Immunization, Centers for Disease Control and Prevention, Atlanta, Ga.

Antibodies. Monoclonal antibody (MAb) M1020, an antibody specific for an activation epitope on CD11a, was obtained from Sanbio Corporation, Uden, The Netherlands (catalog no. M1020). The complete characterization of this antibody has been described elsewhere (16). An MAb to the MV H protein was obtained from Chemicon, Temecula, Calif. (catalog no. MAB 8905).

MV H protein. Partially purified MV H protein (>90% purity as determined by Western blot [immunoblot] analysis and MV-specific antisera) was acquired from Whittaker Bioproducts (catalog no. 30-811J). This protein was prepared from the Edmonston strain of MV. The total protein concentration of the preparation was determined to be 80 mg/ml by using a protein assay kit purchased from Bio-Rad, Richmond, Calif. (catalog no. 500-0001).

Synthetic oligopeptides. Synthetic oligopeptides EZOP, EMOP, MV03, and MV04 with sequences homologous or heterologous to those found in the H protein of the E-Z and E-M strains of MV used in these studies (Table 1) were synthesized by Bio Synthesis Inc., Dallas, Tex. Each peptide preparation was shown to be >95% pure by high-pressure liquid chromatography and free of detectable endotoxin contamination by using a *Limulus* amoebocyte lysate assay kit (pyrogen kit, catalog no. N289; Whittaker Bioproducts).

Virus quantification. Virus in inocula and in leukocyte suspensions was quantified in 96-well tissue culture plates (catalog no. 3072; Falcon, Lincoln Park, N.J.) as described in detail previously (35). The amount of virus in each sample was expressed as the quantity of virus that could infect 50% of inoculated tissue culture monolayers.

Infection of leukocyte cultures. Leukocyte suspensions were adjusted to a concentration of 10⁶ cells per ml, pelleted, and then infected with the appropriate virus, using a multiplicity of infection (MOI) of 0.1. After a 90-min adsorption period with occasional mixing, the cells were washed, resuspended in 10 ml (final concentration, 10⁵ cells per ml) of 10% FCS-RPMI, and placed in 25-cm² tissue culture flasks (catalog no. 3013; Falcon). These flasks were incubated vertically in a 36°C CO₂ incubator for 7 days. On days 0, 2, 4, and 7, two 0.1-ml samples were removed from each flask and centrifuged at 400 × g. The resulting supernatants and pellets were assayed for virus as described in reference 35.

Leukocyte aggregation assay. The nature and kinetics of the leukocyte aggregation induced by MV infection, and the assay used to assess it, have been described in detail previously (4). Aggregation was quantitated by the method of Kansas and Tedder (15), with the exception that the values obtained were expressed as percentages of cells aggregated. In these assays, 10 microscopic fields of each suspension were observed with a 40× objective. An aggregate was defined as ≥3 attached cells. As suggested by the homogeneous single-cell suspension shown in Fig. 1A, such aggregates were not commonly observed in uninfected cultures.

Assays assessing the effects of MV H protein on aggregation were performed in 12-well tissue culture plates (Falcon 3043). The concentration of H protein need to induce changes in LFA-1 expression and leukocyte aggregation were determined in preliminary experiments (e.g., Fig. 3). In experiments examining the effects of different MABs on H protein-induced changes in LFA-1 expression and aggregation, the MABs were added just prior to the addition of the H protein.

Flow cytometric analysis. Expression of LFA-1 on uninfected and MV-infected U937 cells was measured by indirect immunofluorescence staining and flow cytometry. In the primary staining step, the mock-infected and virus-infected cells were incubated at 4°C for 30 min with either a murine isotype control MAb (immunoglobulin G1 [IgG1]; catalog no. 6602398; Coulter Immunology, Hialeah, Fla.) or MAb M1020. The cells were then washed and stained with fluorescein isothiocyanate-labeled goat IgG Fab' with specificity against mouse Fc (catalog no. 55517; Cappel, Cochranville, Pa.). Stained leukocytes were analyzed on an Epics Profile flow cytometer (Coulter Cytometrics, Hialeah, Fla.) by gating on the respective populations and using forward and side light scatter. In each experiment, the cursor for the negative population of cells was set by using the mouse isotype control MAB. The results were expressed as the mean fluorescence intensity.

Fluorescence labeling of oligopeptides for binding assays. Reagent for labeling oligopeptides was prepared by dissolving 1.5 mg of carboxyfluorescein succinimidyl ester (CFSE; catalog no. C-1311; Molecular Probes, Eugene, Ore.) in 1 ml of anhydrous dimethyl sulfoxide. One hundred microliters of this reagent was then added to 1 ml of phosphate-buffered saline (PBS) containing 1 mg of the appropriate MV oligopeptide. These suspensions were then incubated in the dark at 4°C with slow and continuous stirring. After 1 h, labeled peptide was separated from unbound CFSE by dialysis against PBS (24 h with three changes). The retentate was clarified by centrifugation (1,000 × g for 10 min) and then filtered through a 0.22-µm-pore-size filter. The concentration of labeled peptide in each preparation was calculated by using the following formula: peptide concentration (mg/ml) = [OD₂₈₀ - (0.35 × OD₄₉₅)]/1.4, where OD₂₈₀ and OD₄₉₅ are optical densities at 280 and 495 nm, respectively. The fluorescence/peptide (F/P) ratio of each preparation was determined by using the following formula: F/P ratio = [3.3 × OD₄₉₅]/(OD₂₈₀ - (0.35 × OD₄₉₅)). It was determined by using these formulas that the F/P ratio for the CFSE-labeled EZOP oligopeptide was 5.3, while that of EMOP was 4.2. Labeling did not appear to significantly alter the binding capacities of these oligopeptides since in competition assays (discussed below), no indication of inhibition was observed until at least a 10-fold excess of the unlabeled protein was added and complete inhibition was not seen until 400-fold excess unlabeled protein was added.

Measurement of oligopeptide binding affinity. The amount of CFSE-labeled EZOP and EMOP needed to bind all available receptors on U937 cells (i.e., to saturate) was determined by exposing the U937 cells to different concentrations of the CFSE-labeled peptides for 30 min and then washing the exposed cells in PBS supplemented with 15 mM Ca²⁺ and Mg²⁺. The amount of bound fluorescence was determined with a flow cytometer. Gating of the cells was done by using untreated U937 cells, and the values were expressed as fluorescent channel numbers. In blocking experiments designed to determine the specificity of the binding, the U937 cells were treated for 20 min with a 400-fold excess of unlabeled peptide prior to addition of labeled peptide.

Competitive inhibition aggregation assays. Competitive inhibition aggregation assays to determine the abilities of the different oligopeptides to block MV H protein-induced changes in LFA-1 expression and leukocyte aggregation were initiated by adding 2 × 10⁵ U937 cells to V-bottom tubes and centrifuging the tubes at 400 × g. After the supernatant was removed, the appropriate oligopeptide (e.g., EZOP) was added to the pellets at a concentration of 1 mg of purified peptide per 10⁶ cells. After the cell suspensions were incubated on ice for 60 min, the cells were washed with PBS and resuspended in 10% FCS-RPMI to give a

FIG. 1. Comparison of the appearance of suspensions of uninfected U937 monocytoid cells (A) with that of U937 cells in cultures for 4 days and infected with VV (B), rVV-F (C), or rVV-H (D).

final cell concentration of 10^5 cells per ml. The resulting suspensions were then transferred to wells of 12-well tissue culture plates. Medium or MV H protein at a 1:100,000 dilution (final concentration, 0.8 μ g/ml) was then added to the appropriate wells. The plates were incubated at 37°C and observed daily for 3 days for aggregation.

Statistics. Determination of descriptive statistics and Student's *t* test (used to compare the means obtained in each experiment) were performed by using True Epistat, a statistical program designed by T. L. Gustavson of Epistat Services, Richardson, Tex., for IBM-compatible computers.

RESULTS

Homotypic aggregation is induced by rVV-H but not VV or rVV-F. The photomicrographs in Fig. 1 compare the appearances of cell suspensions from one of several experiments examining the formation of leukocyte aggregates (defined as ≥ 3 cells in contact) in uninfected or VV-infected cultures of U937 cells. As indicated by the total lack of contact of the cells in the suspensions shown in Fig. 1A (uninfected U937 cell culture), Fig. 1B (U937 cells infected for 4 days with VV), and Fig. 1C (U937 cells infected for 4 days with rVV-F), homotypic aggregation was rarely seen in healthy, uninfected U937 cell cultures or in cultures infected with VV or rVV-F (using an MOI of 0.1 and an observation period of 7 days). In contrast, as indicated by the large numbers of extensive aggregates seen in Fig. 1D (U937 cells infected for 4 days with rVV-H), homotypic aggregation occurred in all experiments in cultures of U937 cells infected with rVV-H. As a control, Vero cells and HEP-2 cells grown in suspension were infected with rVV-H. No significant aggregation was seen in these cultures over a 7-day observation period (data not shown).

The aggregates observed in rVV-H-infected cultures resembled those formed following MV infection with the exception that no giant cells were ever seen (photomicrographs of MV-induced aggregates and giant cells can be seen in reference 4). The failure of VV and rVV-F to induce aggregation was not due to lack of virus growth, as the kinetics of replication for all three VV constructs were generally similar in the different experiments (in this experiment, for example, all three viruses had maximal titers on day +4 ranging from 5.6 to 5.8 $\log_{10}$ /ml).

MAbs specific for either MV H protein or LFA-1 inhibit the

FIG. 2. Comparison of the mean number of leukocyte aggregates seen on different days in cultures of U937 monocytoid cells infected with rVV-H (slanted hatched bars) with the mean number of aggregates seen in similarly infected cultures into which an MAb specific for MV H protein (vertically striped bars) or for an activation epitope on the CD11a chain of LFA-1 (i.e., MAb M1020; cross-hatched bars) was added. The reduction in mean number of aggregates seen on days 2 and 4 in cultures containing either MAb was significant ($P < 0.01$) compared with the mean number of aggregates in the control cultures lacking antibody, using Student's *t* test.

aggregation induced by rVV-H. Figure 2 compares the mean number of aggregates seen on different days in cultures of U937 cells infected with rVV-H with the mean number seen in similarly infected cultures into which a MAb specific for MV H protein or for an activation epitope on the CD11a chain of LFA-1 (i.e., MAb M1020) was added. As indicated by the low heights of all bars on day 0, aggregate formation was minimal on day 0 in all test cultures. However, by day +2, significant numbers of aggregates were evident in cultures lacking added antibodies, and by day +4, hundreds of large aggregates were present in these cultures. In contrast, minimal numbers of aggregates were seen in cultures containing either MAb on any test day (on days +2 and +4, the mean numbers of aggregates in cell suspensions containing a MAb were all significantly less [$P \leq 0.01$] than the mean number of aggregates seen on these days in cultures lacking added antibodies). With neither antibody was the inhibition of aggregation caused by inhibition of virus replication; equivalent levels of rVV-H were obtained from all cultures on each day (data not shown).

Partially purified MV H protein induces aggregation of U937 cells in a dose-dependent manner. As shown in Fig. 3, aggregation of U937 cells could also be induced by adding partially (>90%) purified MV H protein to cultures of these cells. The aggregation induced by this preparation was dose dependent. Thus, the maximal amount of aggregation seen in these tests occurred when the highest concentration of MV H tested (10 μ g/ml) was added to the cultures, diminished when 10-fold less MV H protein (1 μ g/ml) was added, and was minimal (but still statistically significant [$P < 0.05$ compared with the mean number of aggregates seen in cultures to which no MV H was added]) when only 0.1 μ g of MV H per ml was added. Unlike the aggregation of U937 cells induced by MV or rVV-H, which was not usually evident on the first day of observation (using an MOI of 0.1), the aggregation induced by the two higher doses of partially purified MV H protein was evident within 4 h of the addition of the H protein.

FIG. 3. Dose-dependent induction of aggregation of U937 cells by partially purified MV H protein. The mean numbers of aggregates seen in cultures containing ≥ 0.1 µg of H protein per ml were all significantly greater than the mean numbers of aggregates seen in control cultures to which MV H protein was not added (all *P* values were ≤ 0.05 by Student's *t* test).

As with the aggregation induced by rVV-H (Fig. 2) or certain MV strains (36), addition of MAb M1020 or a MAb specific for MV H protein just prior to the addition of the partially purified MV H protein inhibited virtually all aggregation (data not shown).

Expression of LFA-1 on the surface of U937 cells infected with rVV-H or exposed to MV H protein is increased. Table 2 compares the mean fluorescence values for suspensions of uninfected and VV-, rVV-F-, or rVV-H-infected U937 cells fluorescence stained by using MAb M1020 in the primary staining step. Fluorescence significantly greater than that seen in control suspensions was observed only in samples removed from suspensions infected with rVV-H (i.e., 11.36 versus 7.74). Mean fluorescence values for samples from cultures infected with rVV (8.25) and rVV-F (8.29) were not significantly greater than the mean fluorescence value for the control cultures (7.66) and were not significantly different when compared by Student's *t* test (*P* > 0.05 for both comparisons). Similar increases in mean fluorescence were seen during flow cytometric analysis of U937 cells exposed for 4 h to semipurified (1 mg/ml) MV H protein and stained by using MAb M1020 (11.28 log mean fluorescence for cells exposed to MV H versus 7.74 for control cultures not exposed to this protein).

Comparison of the nucleotide sequences of the H protein genes of the selected measles viruses. Table 3 compares the differences that are known to exist in the nucleotide sequences

TABLE 2. Expression of LFA-1 on uninfected U937 cells, VV-infected U937 cells, or U937 cells exposed to the H protein of MV^a

Antibody	Log mean fluorescence/channel in U937 cells infected or exposed to:				
	No virus	VV	rVV-F	rVV-H	MV H protein
IgG1	0.99	1.12	0.94	0.85	1.02
M1020	7.71	8.25	8.29	11.36	11.28

^a U937 cells were stained 48 h after virus infection (MOI of 0.1) or 4 h after the addition of measles virus H protein, using the control MAb (IgG1) or MAb M1020 (anti-CD11a; IgG1) as the primary antibody. Fluorescence was analyzed on a flow cytometer.

TABLE 3. Comparison of nucleotide substitutions in the H protein genes of several MV vaccine strains relative to the WT Edmonston strain^a

Nucleotide position	Nucleotide at this position in:				Amino acid change
	WT	E-M	E-S	E-Z	
137	C	T	T	T	S→F
351 ^b	C	A	A	C	F→L
631	A	G	G	G	S→G
1441	A	T	T	T	N→Y
1636	G	A	A	A	G→S

^a Adapted from reference 32.

^b The only site which is unique to MV E-M and E-S that is not found in either the E-Z or WT strain.

of the H protein genes of WT Edmonston MV and three MV vaccine strains, E-M, E-Z, and Edmonston-Schwarz (E-S), that were derived from the WT virus (32) and used in these studies. As the limited number of differences shown indicates, there is much homology between the H protein genes of these strains. Closer examination reveals that the nucleotide sequences in the H protein gene of the E-M and the E-S strains are identical and that both of these strains contain a nucleotide at position 351 which is not found in either the E-Z or WT MV strain. This change in nucleotide leads to an amino acid substitution at position 116 from the amino terminus (i.e., phenylalanine in the H protein of the WT strain is changed to leucine in the H protein of the E-M and E-S strains). None of the other differences in bases shown (i.e., at positions 137, 631, 1441, and 1636) are unique to the E-M and E-S strains. Although the nucleotide sequences of the WT and E-Z MV H proteins are not identical, they are homologous at position 351 (amino acid 116).

Synthetic oligopeptides can inhibit the induction of aggregation induced by MV H protein in a competitive inhibition assay. Synthetic oligopeptides homologous and heterologous to the region of the H glycoprotein encompassing amino acid 116 were synthesized and used in a competitive inhibition assay to determine their effects on MV H protein-induced aggregation of U937 cells. As shown in Fig. 4, neither of the two synthetic oligopeptides used in this study (i.e., the EMOP oligopeptide homologous to MV E-M or the EZOP oligopeptide homologous to MV E-Z) induced significant aggregation of U937 cells (*P* > 0.05 compared with the mean aggregation seen in control cultures), even when added to cultures of U937 cells at relatively high concentrations (1 mg/ml). As in previous experiments, marked aggregation of the U937 cells was seen in cultures to which only MV H protein was added. Similar aggregation was seen in suspensions to which EZOP was added prior to the addition of MV H protein, indicating that this peptide could not block H protein-induced up-regulation of LFA-1. However, significantly reduced (*P* < 0.001) homotypic aggregation was observed in suspensions to which EMOP was added prior to the addition of MV H protein. It should be noted that in these experiments, control oligopeptides homologous to sequences of the H protein not encompassing amino acid site 116 (MV03 and MV04 [Table 1]) neither induced aggregation nor blocked that induced by MV H protein, although both of these peptides were tested at the same concentrations as EMOP and EZOP (i.e., 1 mg/ml; data not shown).

Oligopeptides EMOP and EZOP bind to U937 cells. To rule out the possibility that the differences in inhibition of aggregation seen in the competition assay described above were not due to dissimilarities in the binding of the oligopeptides to

FIG. 4. Comparison of the mean number of aggregates seen in cultures of U937 cells incubated with the EZOP or EMOP oligopeptide with the mean number of aggregates seen in U937 cell cultures to which partially purified MV H protein (H pro) or a combination of H pro and EZOP or EMOP was added. When mixtures were added, the oligopeptides were added 60 min before the addition of H pro.

U937 cells, the binding affinities of EZOP and EMOP to U937 cells were determined by using fluorescence-labeled peptides and flow cytometry. As indicated by the curves presented in Fig. 5, both of the peptides bound comparably to U937 cells at each of the different concentrations tested. Moreover, saturation binding for both the peptides occurred at concentrations of between 10 and 20 $\mu\text{g/ml}$. The specificity of the fluorescence seen in these assays was indicated by the fact that addition of a 10-fold excess of homologous unlabeled peptide prior to the addition of labeled peptide partially inhibited fluorescence,

FIG. 5. Comparison of the binding of the fluorescence-labeled ELOP oligopeptide to U937 cells (solid line) with the binding of the fluorescence-labeled EMOP oligopeptide (dashed line). The U937 cells were incubated with different concentrations of CFSE-labeled oligopeptide for 30 min at room temperature, and the fluorescence of the labeled cells was measured on a flow cytometer.

while a 400-fold excess completely inhibited fluorescence (data not shown).

DISCUSSION

This study was instituted to determine the mechanism(s) involved in MV-induced modulation of LFA-1 expression, using an MV-leukocyte model that has been well described (4, 36). Aggregation of U937 cells was used as a convenient means to monitor changes in LFA-1 expression, since it had been shown in the earlier studies that both U937 cells and human peripheral blood leukocytes had equivalent changes in LFA-1 expression following infection with MV and that aggregation always accompanied up-regulation of LFA-1 expression *in vitro*. The use of aggregation to monitor MV-induced changes in LFA-1 expression also appeared appropriate since the aggregation seen *in vitro* appeared to be a correlate of what is thought to occur *in vivo* (i.e., that increased expression of LFA-1 on leukocytes *in vivo* leads to the enhanced adherence of the infected leukocytes to vascular endothelium and subsequently to increased extravasation of these cells and dissemination of virus).

The need to focus on the H protein of MV became apparent after the first experiments examining the effects of VV, rVV-F, and rVV-H on homotypic aggregation. The results obtained clearly showed that of these viruses, only rVV-H could induce significant homotypic aggregation of U937 cells (Fig. 1), although all of the test viruses could grow equally well in U937 cultures. Equally important, the aggregation induced by rVV-H was inhibited by an MAb specific either for epitopes on the H protein of MV or for an activation epitope on the CD11a chain of LFA-1 (i.e., MAb M1020; Fig. 2). These results suggested that both MV H protein and LFA-1 were involved in the aggregation seen.

Additional evidence that the H protein of MV was initiating homotypic aggregation of leukocytes was provided by the demonstration that >90% purified MV H protein could induce aggregation of U937 cells in a dose-dependent manner (Fig. 3). The aggregation induced by the MV H protein differed from that induced by live virus only in that no giant cells were seen (4) and aggregation occurred more rapidly than with live virus (i.e., within 4 h rather than ≥ 24 h). Presumably it takes the virus longer to induce homotypic aggregation than it takes the H protein because the virus requires about 24 h to replicate and produce enough H protein to induce aggregation (using an MOI of 0.1). Undoubtedly, the failure to see giant cells in the aggregates induced by either rVV-H or the H protein was due to the fact that neither this virus nor the protein has F protein, which mediates live virus-induced fusion. Although it can be argued that non-H protein contaminants in the preparation used in these studies were responsible for the aggregation seen, the fact that the MAb specific for epitopes on the H protein almost totally abrogated the response suggests that the aggregation was initiated by the H protein.

Verification that changes in LFA-1 expression were occurring concomitantly with the aggregation induced by either rVV-H or the partially purified H protein preparation was obtained in flow cytometric studies. As indicated by the mean fluorescence values shown in Table 2, both rVV-H and the partially purified MV H protein preparation induced significant increases in the expression of the activation epitope LFA-1 on U937 cells following the addition of these reagents to the U937 cell suspensions. The link between MV H protein and LFA-1 modulation was strengthened by the fact that no significant increase in LFA-1 expression was seen following infection with either VV or rVV-F.

Evidence that a relationship between MV H protein and LFA-1 modulation may exist comes from published gene nucleotide sequences for the three MV vaccine strains used in these studies and for WT MV. As indicated by the data shown in Table 3 (taken from reference 32) and discussed above, there is complete homology in the nucleotide sequences of the H genes of the E-M and E-S strains and only one mutation which is unique to these viruses that is not found in the E-Z or WT Edmonston strain. This mutation is located at nucleotide position 371 from the 5' end and results in a substitution at position 116 of phenylalanine for leucine. The other four mutations in E-M and E-S H protein genes, compared with WT Edmonston, are also present in the E-Z strain. Thus, the presence of the aromatic amino acid phenylalanine at amino acid site 116 appears to correlate with the ability of MV to up-regulate expression of LFA-1 on leukocytes. We, of course, do not know if the nucleotide sequences of all WT MV isolates are identical. However, it is known that there is much homology among these viruses and that all of the WT MV isolates that we have tested for the ability to modulate LFA-1 expression and induce aggregation behave similarly.

More substantial support for the hypothesis that site 116 of the H protein is involved in LFA-1 modulation comes from results of competitive inhibition assays using oligopeptides with sequences encompassing this region and which are homologous or heterologous to regions of the H proteins of MV E-M or E-Z. Although the two primary oligopeptides (i.e., EMOP and EZOP) differed in only one amino acid (underlined in Table 1; phenylalanine was changed to leucine as is seen in the native protein), markedly different results were obtained. In these assays, it was determined (i) that neither oligopeptide could induce homotypic aggregation and (ii) that the EMOP but not the EZOP oligopeptide could block LFA-1-dependent aggregation induced by partially purified H protein. On the basis of these findings, the model shown in Fig. 6 was developed. As indicated, we envision the following: (i) EMOP and EZOP can attach to receptors on the surface of target cells. (ii) Attachment by these peptides cannot induce aggregation, presumably because they are too short or lack the proper configuration to properly cross-link surface receptors. (iii) However, attachment of the E-M or E-Z oligopeptide to its binding site can prevent proper attachment of the H protein to that site. (iv) Binding of the E-Z oligopeptide to its site complements subsequent H protein binding to the relevant binding sites. This leads to sufficient cross-linking or an adequate number of bound sites to initiate activation of LFA-1 and aggregation of U937 cells (i.e., there is no apparent inhibition of aggregation). On the other hand, we envision that attachment of the E-M oligopeptide prevents proper attachment of the H protein at many sites, and since it does not complement H protein binding, there is a reduction in cross-linking or the number of sites bound. The end result of this situation is reduced aggregation of cells, and inhibition is observed. Presumably proper cross-linking of host cell receptors on the cell surface by the MV H protein leads to a sequence of intracellular events (i.e., signaling) which mediate the alteration of LFA-1.

In these studies, we have emphasized the potential of MV-induced modulation of LFA-1 expression to increase the pathogenesis of this virus. However, this activity may also influence the immunogenicity of MV and MV vaccines. For example, the MV E-Z vaccine strain has been reported to have superior immunogenicity and protective efficacy in babies less than 9 months old (1, 20, 34) compared with the protection conferred by standard MV vaccine strains. Although it is generally thought this increased immunogenicity is due to use of

FIG. 6. Proposed model to explain how the EZOP and EMOP oligopeptides can block, or fail to block, MV H protein-induced homotypic aggregation in cultures of U937 monocytoid cells in competitive inhibition assays (see Fig. 4, Results, and Discussion for more detail).

higher-titered vaccine, it is possible that the enhanced ability of the E-Z vaccine strain to infect peripheral blood mononuclear leukocytes and evade the neutralizing activity of MV-specific antibodies is what is responsible for the increased immunogenicity and protective efficacy of this vaccine in infants with significant levels of maternal antibodies.

Further studies with the MV-leukocyte model are planned in hope that understanding of the mechanisms that mediate MV-induced modulation of LFA-1 on leukocytes can lead to methods to intervene and reduce virus dissemination and immune dysfunction induced by this and other leukotropic viral pathogens (10). These studies may also contribute to our understanding of how to make vaccines more immunogenic.

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