

Immunoglobulin G Antibody-Mediated Enhancement of Measles Virus Infection Can Bypass the Protective Antiviral Immune Response

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Antibodies to viral surface glycoproteins play a crucial role in immunity to measles by blocking both virus attachment and subsequent fusion with the host cell membrane. Here, we demonstrate that certain immunoglobulin G (IgG) antibodies can also enhance the entry of measles virus (MV) into monocytes and macrophages. Antibody-dependent enhancement of infectivity was observed in mouse and human macrophages using virions opsonized by a murine monoclonal antibody against the MV hemagglutinin (H) glycoprotein, polyclonal mouse anti-MV IgG, or diluted measles-immune human sera. Neither H-specific Fab fragments nor H-specific IgM could enhance MV entry in monocytes or macrophages, indicating involvement of a Fc γ receptor (Fc γ R)-mediated mechanism. Preincubation with an anti-fusion protein (anti-F) monoclonal antibody or a fusion-inhibitory peptide blocked infection, indicating that a functional F protein was required for viral internalization. Classical complement pathway activation did not promote infection through complement receptors and inhibited anti-H IgG-mediated enhancement. In vivo, antibody-enhanced infection allowed MV to overcome a highly protective systemic immune response in preimmunized IfnarKo-Ge46 transgenic mice. These data demonstrate a previously unidentified mechanism that may contribute to morbillivirus pathogenesis where H-specific IgG antibodies promote the spread of MV infection among Fc γ R-expressing host cells. The findings point to a new model for the pathogenesis of atypical MV infection observed after immunization with formalin-inactivated MV vaccine and underscore the importance of the anti-F response after vaccination.

Measles is considered the most contagious human infectious disease known, affecting mainly young infants. *Measles virus* (MV) belongs to the family *Paramyxoviridae* and carries a negative-sense RNA genome incorporated in a helical ribonucleoprotein complex and packaged in a lipoprotein envelope (12). Immunization with the live attenuated measles virus vaccine has dramatically reduced the number of infections, but still more than 30 million cases, 0.5 to 0.7 million of them fatal, are reported annually (19, 27, 38). Two cellular glycoproteins, the membrane cofactor protein CD46 and CD150, also known as SLAM or signaling lymphocytic activation molecule, have been identified as MV receptors (25, 46). Viral attachment and entry into the host cells are mediated by two surface glycoproteins—hemagglutinin (H) and fusion protein (F) (12, 20, 41). MV is spread by aerosol and initiates its replication cycle in the epithelial cells of the upper airway. During the 10- to 14-day incubation period, the infection extends to the regional lymph nodes and via the bloodstream reaches a variety of distant organs, including lymph nodes, spleen, liver, lung, thymus, and skin. Usually, after a 2- or 3-day period of nonspecific prodromal symptoms, patients develop the typical maculopapular rash and Koplik's spots. Protective antibodies and cell-mediated immune response are apparent at the time of onset of the rash and are responsible for complete recovery and lifelong immunity against reinfection. Measles characteristically causes transient immune suppression with leukopenia, altered cyto-

kine secretion, and a high incidence of secondary opportunistic infections (22). Many of the normal functions of macrophages and dendritic cells are impaired as part of the generalized immunosuppression caused by MV (17, 42). Imbalanced production of proinflammatory molecules, including suppression of tumor necrosis factor alpha and interleukin 12 (IL-12), and elevated production of IL-10 and IL-4 have been proposed to explain the characteristic Th1-Th2 alterations that are seen in MV infection and that lead to skewing of the immune response (21, 37).

An unexpectedly severe form of measles (atypical measles) was observed in immunized persons in the mid-1960s when formalin-inactivated measles vaccine (FIMV) was applied (8). Three doses of FIMV induced a short-lasting humoral immune response, followed several months or years later by susceptibility to atypical measles, a prolonged illness characterized by high fever with unusual petechial or vesicular skin lesions. Severe pneumonitis with nodular infiltrations, lymphadenopathy, and pleural effusions were the major complications requiring hospitalization (12). Many hypotheses have been advanced to explain the pathogenesis of atypical measles, including imbalance between H- and F-specific antibodies, immune complex deposition, and the absence of protective cell-mediated immunity (35, 36).

MV is also emerging as a promising agent for cancer virotherapy. MV vaccine strains can selectively and destructively propagate in tumor cells without significantly damaging healthy tissue (13, 28, 29, 34). Clinical activity has been demonstrated after intratumoral administration in T-cell lymphoma (15), and additional clinical trials are under way or planned for treating ovarian cancer, glioma, and multiple my-

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eloma (MM). Preexisting measles immunity, whether the result of immunization or natural infection, is expected to significantly impact the outcome of oncolytic MV therapy in these studies. Thus, the effects of neutralizing antibodies and other adaptive immune mechanisms require additional investigation.

Here we demonstrate for the first time *in vitro* and in susceptible transgenic mice the crucial role of antibody-dependent enhancement of infection within monocytes and macrophages as a mechanism whereby MV can “bypass” a preexisting immune response. The results point to an additional pathogenetic mechanism that may be in play during MV infections and which may have particular relevance to the pathogenesis of the atypical measles that is seen after immunization with inactivated vaccine.

MATERIALS AND METHODS

MV strains and virus propagation. Green fluorescent protein (GFP)-expressing MV (MV-GFP) and human carcinoembryonic antigen (CEA)-expressing MV (MV-CEA) were propagated in Vero cells (6, 31). Recombinant MVs completely ablated for the natural receptors CD46 and SLAM (MV-aa-H6 and MV-aals-H6) were also used in this study. All MV strains, including nonablated MV-H6, expressed six histidine residues on H protein that allowed successful rescue and replication in anti-His single-chain antibody displaying Vero-His cells (24). Viral stocks were prepared by repeated freezing-thawing procedures, and MV titer was determined as 50% tissue culture infectious dose (TCID₅₀) using Vero or Vero-His cells.

Human macrophages, cell lines, and hybridomas. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from healthy donor blood samples. Nonadherent cells were removed after incubation for 2 to 4 h in polystyrene plates (Falcon), and attached monocytes were left to differentiate to macrophages in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) with a low endotoxin level (<1 ng/ml) (MP Biomedicals). Human monocytic lines (U-937 and THP-1), ARH-77 cells, and mouse J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The MM cell lines KAS-6/1 and JIN-3 were kindly provided by D. Jelinek and R. Fonseca from the Mayo Clinic, Rochester, MN. Hybridoma clones secreting monoclonal antibodies (MAbs) CL55 (immunoglobulin G2b [IgG2b] isotype) and CL48 (IgM) against MV hemagglutinin and Y503 (IgG1) specific for MV fusion protein were kindly provided by T. F. Wild, Institut Pasteur de Lyon, France. Human cell lines and murine macrophages were maintained in media recommended by ATCC—Dulbecco modified Eagle medium or RPMI 1640 medium with 10% FBS (Gibco) and supplements. KAS6/1 cell growth was supported by adding 1 ng/ml human recombinant IL-6 (R&D Systems). Purified MAbs were prepared by affinity chromatography from supernatant of mouse hybridomas grown in HyQ-CCM1 serum-free medium (HyClone). All cells were grown in a 37°C incubator in a humidified atmosphere with 5% CO₂ (Haereus).

Purification of MAbs and mouse serum IgG and generation of Fab fragments. Serum-free supernatants of IgG2b MAb CL55 and IgG1 MAb Y503 were purified on ImmunoPure immobilized protein A and protein L columns, respectively, according to the manufacturer's protocol (Pierce Biotechnology, Inc.). The eluted MAbs were dialyzed against phosphate-buffered saline (PBS), pH 7.4. The IgM clone CL48 was purified from serum-free supernatant by ammonium sulfate precipitation. Protein concentration of the preparations was determined by absorbance at 280 nm. The purity of each preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. Polyclonal serum IgG from MV-immunized or nonimmunized mice was purified by affinity chromatography on a protein G column (Sigma) or by cold ethanol precipitation and subsequent fractionation on a Superose 6 column (Pharmacia).

IgG antibodies from pooled mouse serum samples were diluted in PBS, filtered, and applied to 1-ml protein G columns (Pierce Biotechnology, Inc.). The columns were washed, and IgG was eluted using binding and elution buffers (Pierce Biotechnology, Inc.). The eluted IgG preparation was dialyzed, lyophilized, and dissolved in PBS. Protein concentration was measured, and purity was confirmed by SDS-PAGE.

Fab fragments were generated from cold-ethanol-purified IgG using an ImmunoPure Fab preparation kit (Pierce Biotechnology, Inc.) following the manufacturer's instruction protocol. Eluted fragments were dialyzed extensively

against PBS and sterilized by filtration through 0.22- μ m filters (Millipore), and protein concentration was measured.

SDS-PAGE and immunoblotting. Samples of purified antibody preparations were mixed 1:1 with reducing (2% 2-mercaptoethanol) or nonreducing loading buffer (Bio-Rad) and boiled for 5 min. For immunoblotting, 5×10^3 TCID₅₀ of MV-GFP or 30 μ g of total protein from 2×10^7 TCID₅₀/ml viral stock (MV-GFP grown on Vero cells) was added directly to an equal volume of Laemmli's buffer containing 2% 2-mercaptoethanol and boiled. Proteins were resolved in Criterion gels (Bio-Rad) using the Criterion electrophoresis system and prestained Kaleidoscope markers from Bio-Rad. Gels were stained with Coomassie brilliant blue R-250 solution (Bio-Rad) and dried.

For immunoblotting, SDS-PAGE-fractionated proteins were transferred to polyvinylidene difluoride (Bio-Rad) or nitrocellulose (Amersham) membranes using a semidry transfer apparatus (Bio-Rad). Membranes were blocked overnight at 4°C in PBS with 0.05% Tween 20 and 2% bovine serum albumin (Sigma) or 5% skim milk. Then the blots were incubated with mouse serum samples diluted (1:250 to 1:2,000) in PBS with 0.05% Tween 20 for 1 or 2 h at room temperature. Specific protein bands were visualized using secondary goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma) and chemiluminescence reagent (Roche).

Human serum samples. Human blood samples were collected from three patients with newly diagnosed MM after Institutional Review Board approval. Sera were heat inactivated by incubation at 56°C for 30 min and tested by virus neutralization (VN) test. Two of these samples, S-1 and S-2, were used for antibody enhancement experiments.

VN assay and VN test in the presence of complement. For VN assay, mouse sera were heat inactivated (56°C for 30 min) and serially diluted in Opti-MEM medium (Invitrogen). Purified IgG and MAbs were applied in different concentrations in Opti-MEM medium. MV-GFP at 50 TCID₅₀ or 50 PFU was mixed with the respective antibodies at various dilutions in 96-well plates and incubated at 37°C for 1 h. Vero cells (10^4 per well) were added, and the number of GFP-positive plaques was counted after 3 days of culture. The data were calculated as the number of PFU/well or as a percentage of the control for residual infection. Plaque reduction neutralization (PRN) titer (50% reduction of the viral input) was determined as described previously (49). Each sample was run in six wells, and each experiment was repeated twice. To assess the role of the classical complement pathway in MV neutralization, 10 to 20% guinea pig or rabbit (both from MP Biomedicals) complement was added to the antibody-virus mixture. VN assays were also run in 24-well plates using 100, 10^3 , and 10^5 TCID₅₀ per well preincubated with antibody dilutions (with or without complement) and inoculated onto 70 to 80% confluent Vero cells.

In vitro infection of monocytes and macrophages. Recombinant MVs were mixed with MAbs in Opti-MEM medium and incubated for different time intervals (from 15 min to 2 h) at 37°C. Adherent mouse J774A.1 macrophages were cultured in 24-well plates (2×10^4 to 5×10^4 cells/well) for 2 days. Prior to infection, monolayers were carefully washed two times with prewarmed Opti-MEM medium. Cells were incubated with the opsonized viruses for different time intervals (15 min to 12 h) at 37°C. Then the inoculum was replaced with fresh Dulbecco modified Eagle medium with 10% FBS. GFP expression in infected cells was examined on days 1, 2, and 3 using a fluorescence microscope (Nikon). MAbs were used at concentrations of 5, 10, and 20 μ g/ml. Purified IgG, Fab fragments, and serum samples were applied in serial dilutions in Opti-MEM medium. The cells were inoculated at different multiplicities of infection (MOIs) from 0.01 to 1 for different time intervals from 30 min to 12 h.

In parallel experiments, J774A.1 cells in suspension were also infected. Briefly, MV was incubated with the antibodies for different time intervals of 15 to 60 min. The cells were harvested by scraping, washed once, and added to the opsonized virus mixture. After incubation for 30 to 60 min, fresh medium was added, and the cells were replated in 24- or 96-well plates (Falcon). For FACS analysis, infected cells were grown in six-well plates.

Counting of individual infected cells by FACS required blockade of cell-cell fusion (2). Fusion-inhibitory peptide (FIP) (Z-D-Phe-L-Phe-Gly-OH [Bachem]) (20 μ g/ml) or MAb Y503 (5 to 10 μ g/ml) was used to inhibit F-protein function.

Maturation of human monocytic lines to macrophages was induced by 10 ng/ml phorbol ester (12-O-tetradecanoylphorbol-13-acetate [TPA]) (Sigma) for 24 to 48 h. The cells were grown to 10^5 per well in 24-well plates. MVs with their natural receptor tropisms ablated (MV-aals-H6) were incubated for 30 min with the MAbs and then added to the cells at final MOIs of 0.01 and 0.1. In parallel, undifferentiated U-937 and THP-1 cells were infected in suspensions as described above.

Human macrophages differentiated from isolated PBMCs were infected in suspension after incubation with opsonized MV-aals-H6. To demonstrate the

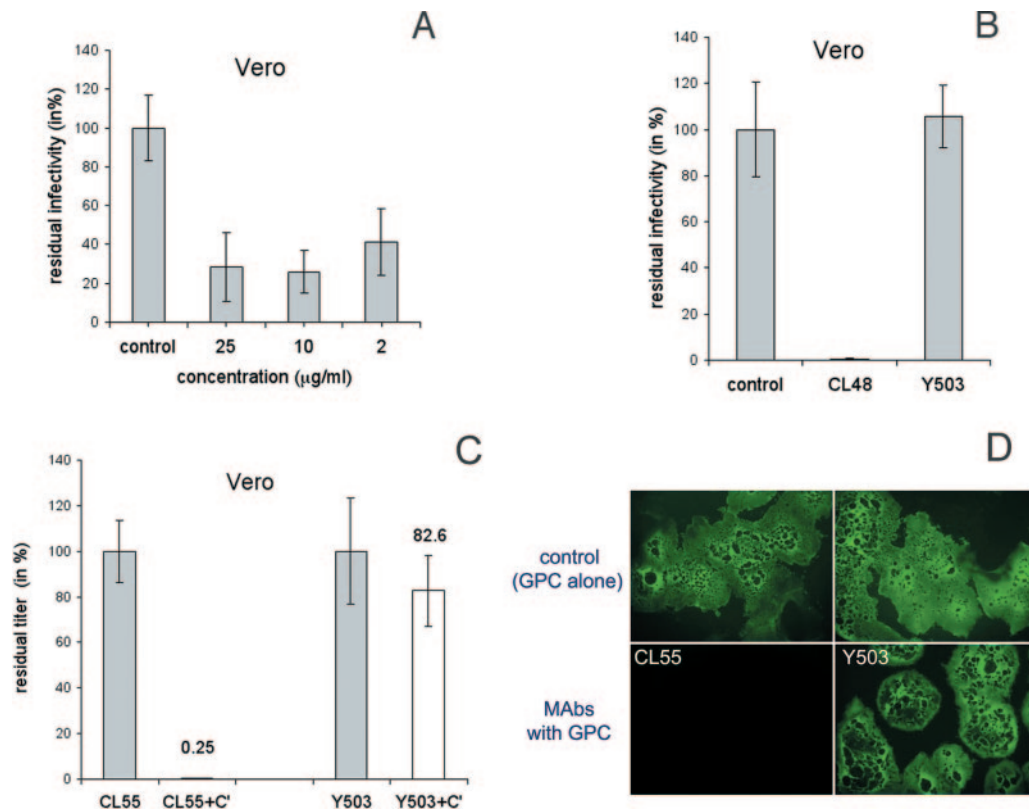


FIG. 1. In vitro neutralizing activity of MV-specific MAbs. The H-specific IgG2b MAb CL55 was preincubated at various concentrations with 100 TCID₅₀ MV-GFP for 1 h and then titrated on Vero cell monolayers on 24-well plates. After 3 days of culture, the number of PFU in each well were counted, and residual infectivity compared to the control without MAb was calculated (A). VN tests (PFU/well) were conducted on Vero cells for IgM MAb CL48 and anti-F IgG1 MAb Y503 at 5 µg/ml (B). Classical complement pathway activation by H-specific MAb CL55 but not by anti-F MAb Y503 resulted in almost complete virus neutralization. MV-GFP (10⁵ TCID₅₀) was incubated for 30 min at 37°C with 5 µg/ml MAbs and 20% GPC. The samples were then diluted and titrated on Vero cell monolayers in 24-well plates (C). No infection was detected after incubation of 10³ TCID₅₀ with CL55 in the presence of GPC (D). In contrast, complement alone and Y503 with 20% GPC could not prevent MV infection. Large MV-GFP-infected syncytial plaques were seen after treatment with complement alone or Y503 plus GPC, but not for samples treated with CL55 plus GPC. C', complement.

roles of the classical complement pathway and complement receptors (CRs) for MV infection in macrophages, the experiment was repeated in the presence of active complement components. The virus and antibodies were incubated for 30 min in the presence or absence of guinea pig complement (GPC). Then the experiment followed the procedure described above. Heat-inactivated or active GPC was used as controls.

To test the feasibility of MV transferring from macrophages to different cell types by heterologous cell-cell fusion, J774A.1 macrophages were infected by IgG-mediated enhancement and exposed for 1 h to MAb CL48 (10 to 20 µg/ml) and 10 to 20% GPC to neutralize unbound virus. After five washes, macrophages were mixed with human MM (KAS-6/1 and JJN3) and lymphoblastoid ARH-77 cells or overlaid on Vero-His cell monolayers.

Flow cytometry (FACScan) analysis. The cells were harvested washed in ice-cold PBS and fixed in 1% paraformaldehyde (Electron Microscopy Sciences). The percentage of infected (GFP-positive) cells was counted on an Excalibur flow cytometer (Becton Dickinson).

In vivo experiments. All in vivo experimental protocols were approved by the Mayo Foundation Institutional Animal Care and Use Committee. Mice were maintained in the barrier facilities of the Mayo Clinic, Rochester, MN.

Immunization of mice and serum collection. IfnarKo-CD46Ge transgenic mouse line—knockout for type I interferon (IFN) receptor and expressing human CD46 receptor—was kindly provided by R. Cattaneo, Mayo Clinic, Rochester, MN (23). Groups of 20 (6- to 8-week-old) animals were injected subcutaneously (s.c.) and intraperitoneally (i.p.) with 2 × 10⁶ TCID₅₀ of MV-GFP. The immunization was repeated four times at weekly intervals, and 7 days after the last application, mice were bled and serum samples were frozen at -80°C. Control sera were collected from animals injected with PBS. The MV-specific

IgG titer was measured by an indirect immunofluorescence test using MV antigen substrate slides (Bion Enterprises) according to the manufacturer's protocol. The neutralizing titer was determined by VN assay as described above, and antibody specificity for viral proteins was confirmed by immunoblotting.

IgG-mediated enhancement of infection in IfnarKo mice. IfnarKo is a type I IFN receptor knockout (*H-2K^K* haplotype) substrain of the IfnarKo-CD46Ge line that has lost CD46 expression. Groups of 7-week-old mice were injected i.p. with 100 µg purified serum IgG fraction diluted in 500 µl sterile PBS. Three days later, animals received 2.5 × 10⁶ TCID₅₀ of MV-CEA in 300 µl PBS via the i.p. route of injection. Four days later, all mice were bled, and the serum levels of human CEA were measured in the Mayo Central Clinical Laboratory, Mayo Clinic, Rochester, MN.

Protective immune response against rechallenge with MV-CEA in CD46 transgenic mice. Groups of 6- to 8-week-old IfnarKo-CD46Ge mice were vaccinated i.p. three times (on days 1, 7, and 14) with 10⁷ TCID₅₀ MV-GFP. On day 35, immunized and control animals were rechallenged by intravenous (i.v.) or i.p. injection of 10⁷ TCID₅₀ MV-CEA. Three days later, mice were bled, and CEA serum levels were determined.

IgG antibody-enhanced infection in preimmunized CD46 transgenic mice. IfnarKo-CD46Ge mice (6 to 8 weeks old) were separated into five groups. Groups 1, 2, and 3 were vaccinated three times with 2 × 10⁶ TCID₅₀ MV-GFP as described above, and groups 4 and 5 served as nonimmunized controls. One week after the fourth vaccination, group 1 (10 animals) received four doses of 60 µg (240 µg total) of purified MV-specific IgG injected i.p. and s.c. at weekly intervals. Groups 2 and 4 (five mice per group) were injected with the same amount of control IgG. Mice from groups 3 and 5 received only PBS. Three days after the last IgG injection, all animals were challenged i.p. with 2 × 10⁶ TCID₅₀

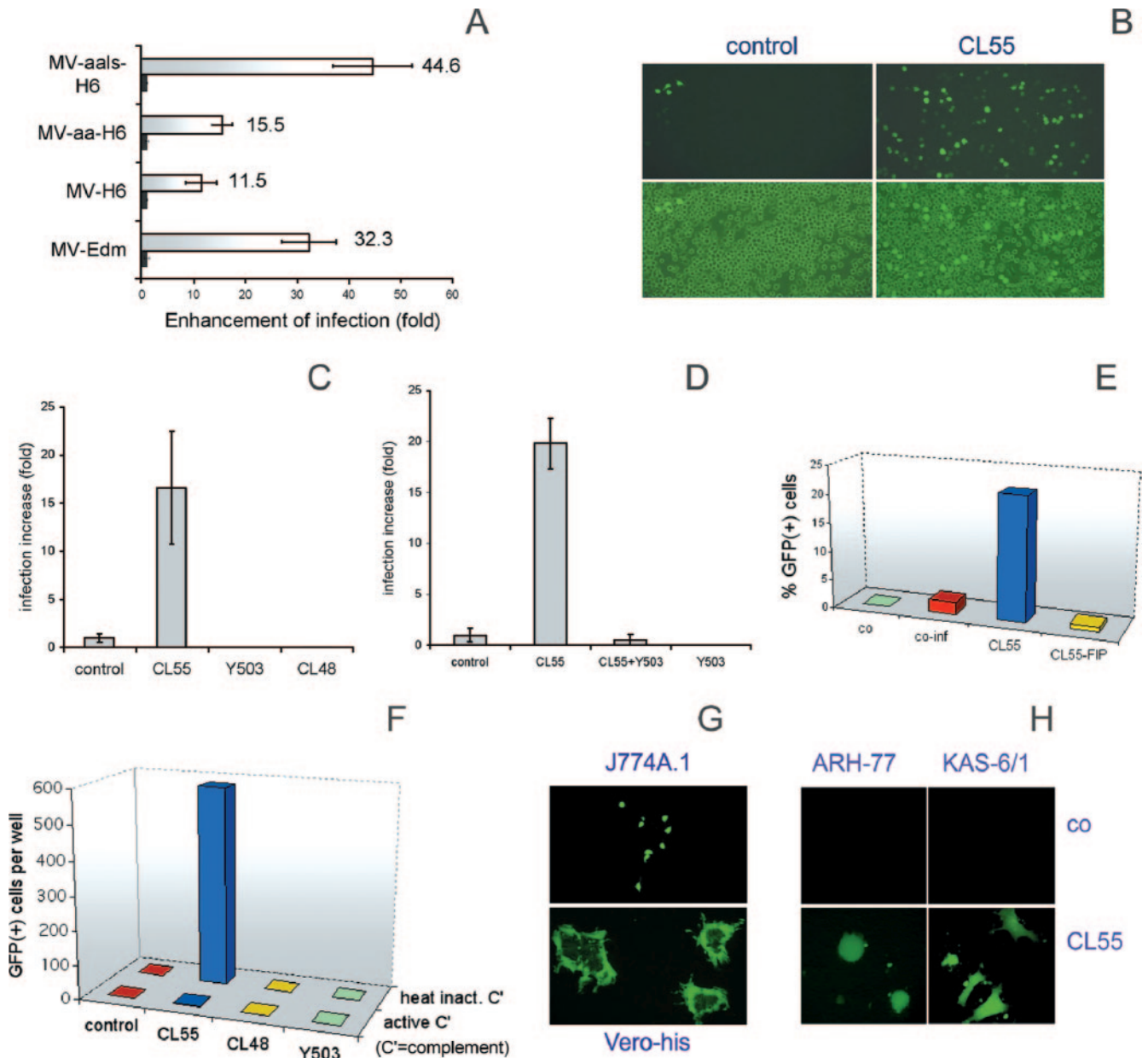


FIG. 2. IgG-mediated enhancement of MV infection in mouse J774A.1 macrophages. (A) CL55 MAb enhances infectivity of different MV strains. GFP-expressing MV strains were preincubated with 5 μ g/ml of the IgG2b MAb CL55 for 30 min and then added to J774A.1 cells at a final MOI of 0.2 or 0.1 (only for MV-H6). The experiments were run in 6 or 12 replicate wells ($P < 0.001$). (B) Infected (GFP-positive) cells after exposure to untreated (control) MV-GFP or MV-GFP pretreated with CL55. (C) In contrast to CL55, IgM MAb CL48 (H protein specific) and IgG1 MAb Y503 (F protein specific) do not promote MV infection in J774A.1 macrophages. MV-aals-H6 was preincubated for 30 min with 5 μ g/ml of each antibody, and the cells were inoculated with a final MOI of 0.2 ($P < 0.001$). (D) Anti-F MAb Y503 completely abolishes CL55 MAb-mediated infectivity enhancement. Both MAbs were used at a concentration of 5 μ g/ml. MV-GFP was incubated for 30 min with CL55 and then for an additional 30 min in the presence of Y503. J774A.1 cells were exposed to the virus for 1 h with a final MOI of 0.5 ($P < 0.001$). (E) FIP (20 μ g/ml) abolishes CL55-mediated (5 μ g/ml opsonization concentration) enhancement of infection. J774A.1 macrophages were exposed to MV-GFP for 12 h at a MOI of 0.5 and later subjected to FACS analysis. The results are expressed as the percentage of GFP-expressing [GFP(+)] cells on day 3 after infection. co, control (noninfected cells); co-inf, control cells infected with nonopsonized virions. (F) Effect of the classical complement pathway activation on CL55-mediated infectivity enhancement in J774A.1 cells. MAbs (5 μ g/ml) and 10% rabbit complement (C') (active and heat-inactivated [heat inact.] control) were incubated with MV-aals-H6 for 1 h and transferred onto adherent macrophages (MOI of 0.2) for 45 min. The number of GFP-expressing cells was counted on day 3 after infection. The results are from a representative experiment with guinea pig or rabbit complement run in six wells of 24-well plates ($P < 0.001$). (G) MV survived antibody- and complement-mediated neutralization within infected macrophages and transferred infection to epithelial cells. Mouse J774A.1 cells were successfully infected by fully retargeted MV-aa-CD38 opsonized with 10 μ g/ml CL55 MAb (top). The cells were treated with MAb CL48 and complement to neutralize all noninternalized virus particles and subsequently were overlaid on Vero-His cell monolayers (bottom). (H) Infected J774A.1 macrophages can also transfer MV-GFP infection to human lymphoblastoid (ARH-77) or MM (KAS-6/1) lines as indicated by the formation of large syncytia. co, control (J774A.1 cells incubated with nonopsonized virions and subsequently treated with MAb CL48 and complement to neutralize all noninternalized virus particles).

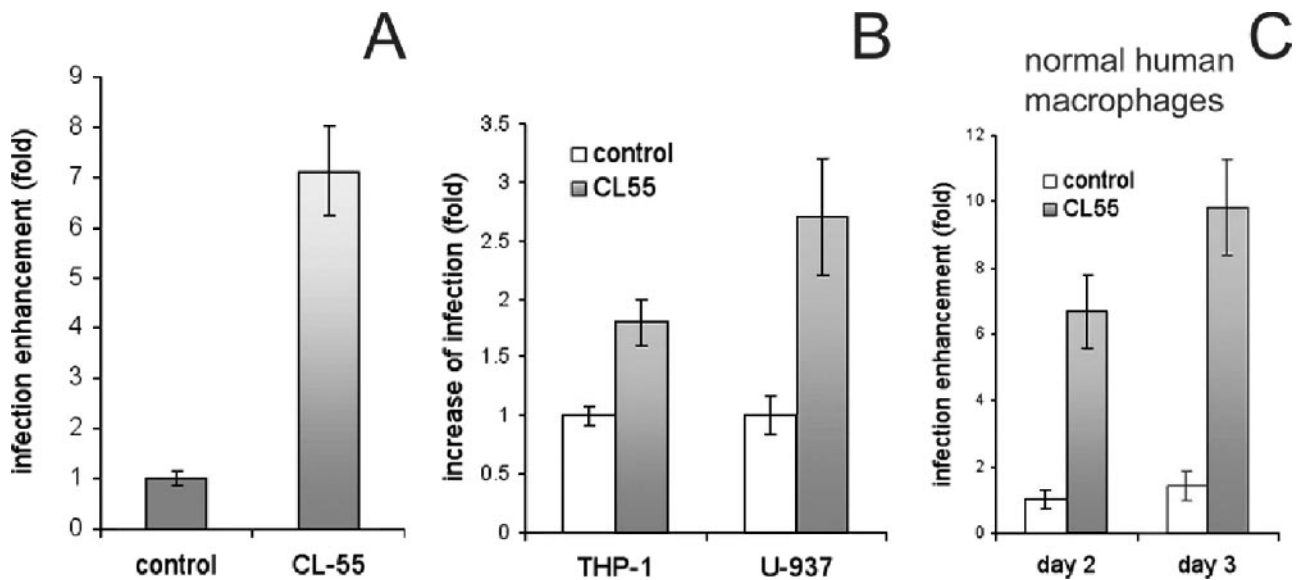


FIG. 3. IgG-mediated enhancement of infection in human macrophages and monocytic cell lines. The effect was demonstrated using MVs ablated for binding to the natural receptors, CD46 and SLAM. (A) IgG-mediated enhancement of MV-aa-H6 infection (MOI of 0.136) in phorbol ester-differentiated U-937 cells. Opsonized viruses (5 $\mu\text{g}/\text{ml}$ of MAb CL55) were incubated with cells for 30 min. The number of GFP-positive cells was counted 3 days after infection ($P < 0.001$). (B) Enhancement of infection in TPA-differentiated THP-1 or undifferentiated U-937 cells infected for 3 h or 30 min, respectively, with MV-aals-H6 (MOI of 0.02 or 0.1, respectively) opsonized by 10 $\mu\text{g}/\text{ml}$ of MAb CL55. Infected cells were counted on day 2 after infection. Data are from representative experiments run in six wells in 24-well plates ($P < 0.001$). (C) Infection of normal (PBMC-derived) human macrophages. MV-aals-H6 was mixed with 10 $\mu\text{g}/\text{ml}$ CL55 for 30 min. The cells were infected for 5 h (MOI of 0.01) and cultured for 3 days in 96-well plates. The results were calculated from one of two independent experiments run in five wells ($P < 0.001$).

of MV-CEA in 250 μl PBS. Seventy-two hours later, serum samples were collected for CEA determination.

Statistical analysis. Statistical analysis was carried out using the Student *t* test. The data obtained are expressed as mean \pm standard deviation (SD). *P* values of < 0.05 were considered statistically significant.

RESULTS

Enhancement of measles virus infection in mouse and human macrophages by a monoclonal antibody to H protein. Hybridoma clone CL55 produces a virus-neutralizing MAb of the IgG2b isotype recognizing an epitope on the H protein of MV (10). In our VN tests, concentrations of 5 to 10 $\mu\text{g}/\text{ml}$ reduced MV-GFP infection of Vero cells by 60 to 88%, confirming its neutralizing activity (Fig. 1A). The H-specific IgM MAb CL48 showed higher protective activity with 99% neutralization at 5 $\mu\text{g}/\text{ml}$. In contrast, the F-specific MAb Y503 (IgG1) at a concentration of 5 $\mu\text{g}/\text{ml}$ for 1 hour did not block infection of Vero cells (Fig. 1B). This antibody was previously reported to inhibit cell-cell fusion but not virus entry in epithelial cells (4). Classical complement activation strongly increased the protective effect of MAb CL55. Thus, 10^5 TCID₅₀ of MV-GFP was almost fully neutralized by 5 $\mu\text{g}/\text{ml}$ CL55 and 20% GPC after 1 h of incubation at 37°C (Fig. 1C and D). Active complement reduced MV infectivity approximately 50 to 70% in the absence of antibodies (not shown), confirming the recently reported role of the alternative pathway against MV (5). In contrast, MV preincubation with the F-specific Y503 antibody in the presence of GPC did not reduce infection in Vero cell monolayers (Fig. 1C and D).

Murine cells are resistant to MV infection, and only a low percentage can be infected even at high MOIs (see controls in

Fig. 2). Incubation of MV with MAb CL55 dramatically enhanced infection in the murine monocytic cell line J774A.1 (Fig. 2A and B). The experiment was repeated more than 20 times with different MV strains, MOIs, and times of incubation, and infectivity was consistently enhanced from 10-fold to more than 50-fold. Up to 21.6% of the monocytic J774A.1 cells could be infected using MV-GFP at a MOI of 1 after a 12-h infection and subsequent FACSscan analysis on day 3 (Fig. 2E). The preincubation time (15 min to 2 h) made no difference to the magnitude of infectivity enhancement. In contrast to CL55, another H-specific MAb CL48 of the IgM isotype could not promote viral entry and even blocked the low level of nonspecific infection seen in mouse macrophages. This result suggested that the enhancement of infectivity seen with CL55 might be mediated via Fc γ receptors (Fc γ Rs) (Fig. 2C). Although the F-reactive clone Y503 was of the IgG1 class, no enhancement of infectivity in J774A.1 cells was observed. This suggested the potential importance of the targeted antigen (H versus F) and the antibody isotype for infectivity enhancement. IgG-mediated infection of macrophages was strongly inhibited when the virus was preincubated with CL55 MAb in the presence of 5 or 10 $\mu\text{g}/\text{ml}$ Y503 (Fig. 2D) or in the presence of 20 $\mu\text{g}/\text{ml}$ FIP (Fig. 2E), indicating that F-protein-mediated fusion is strictly required for infection initiation. Also, no enhancement was seen when virus was preincubated with CL55 MAb in the presence of GPC or with GPC alone. MV escaped from antibody- and complement-mediated neutralization within J774A.1 macrophages, and infection was successfully transferred to epithelial or MM cells (Fig. 2G and H). Large syncytia (equivalent to those seen after direct MV infection) were seen in all three human lines tested, illustrating that monocyte/

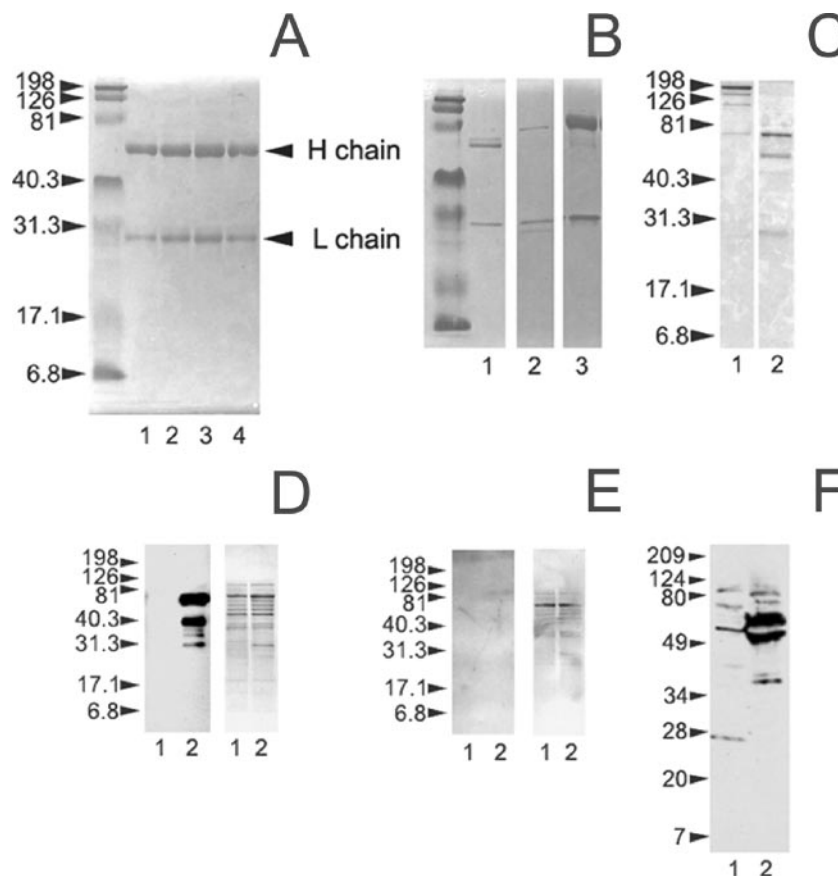


FIG. 4. (A) SDS-PAGE demonstrating the purity of four different IgG preparations isolated from immune mouse serum by affinity chromatography. Proteins were resolved on 12.5% polyacrylamide gels (2 μ g protein per lane). The positions of the marker protein standards (in kilodaltons) are indicated by black arrowheads to the left of the gels. The positions of heavy (H) and light (L) chains are shown to the right in panel A. (B) The purity of the MAb preparations was checked on a 15% polyacrylamide gel. Lane 1, CL55 (1 μ g per lane); lane 2, Y503 (1 μ g per lane), lane 3, CL48 (3 μ g per lane). (C) Cold-ethanol-purified serum IgG was run on a 12.5% polyacrylamide gel under nonreducing (lane 1) and reducing (lane 2) conditions. (D and E) Immunoblotting reaction of MV-specific serum (D) and control serum (E) used for cold ethanol precipitation and subsequent gel chromatography. Lanes 1, control Vero cell lysates; lanes 2, MV antigens. Polyvinylidene difluoride membranes were stained (Coomassie brilliant blue) later to demonstrate the amount of protein per lane (right blots). (F) MV specificity of the affinity chromatography-purified serum IgG was also confirmed by immunoblotting.

macrophages infected by antibody enhancement mechanism could be an efficient MV carrier for in vivo oncolytic virus therapy.

To investigate the phenomenon of antibody-enhanced infection in human monocytes/macrophages, we used a recombinant measles virus ablated in its attachment sites for the natural measles virus receptors CD46 and SLAM (MV-aa-H6 and MV-aals-H6). Opsonization of MV-aa-H6 with 5 to 10 μ g/ml CL55 increased the number of infected U-937 cells up to seven times (Fig. 3A). We observed 1.7- to 3-fold enhancement of MV-aals-H6 infectivity in THP-1 cells and in immature U-937 (no TPA stimulation) (Fig. 3B). As with mouse J774A.1 cells, the presence of antibody plus complement neutralized the virus, and no enhancement was observed with IgM MAb CL48 or with the Y503 F-specific antibody (not shown). MAb CL55 enhanced MV-aals-H6 infection in primary human macrophages 6.7- to 6.9-fold compared to the controls (Fig. 3C).

Enhancement of MV infection in macrophages by mouse and human polyclonal IgG antibodies and anti-MV sera. To investigate the existence and potential relevance of antibody-

enhanced infection in natural (polyclonal) anti-MV antibody responses, we used purified antibodies and dilute anti-MV sera. Protein concentrations and purity of all antibody preparations (including MAbs) were demonstrated by SDS-PAGE under reducing and nonreducing conditions (Fig. 4A to C). Pooled serum samples from transgenic mice immunized i.p. with MV showed high neutralizing activity with a complete neutralization titer of 1:1,280 and a PRN titer of $>1:10^4$, but the cell-cell fusion inhibition titer was $<1:4$. Purified IgG from these animals could completely inhibit infection at 25 μ g/ml (PRN titer of <5 μ g/ml), but cell-cell fusion was resistant to neutralization even at concentrations of >1 mg/ml (Table 1). Reaction of each antibody preparation with MV antigens was confirmed by immunoblotting (Fig. 4D to F).

Polyclonal IgG from immunized mice enhanced MV infection in J774A.1 cells between 8- and 30-fold (for the different experiments) compared to polyclonal IgG from control mice (Fig. 5A and B). The antibodies were equally effective whether purified by affinity chromatography or by cold ethanol precipitation. Unfractionated serum samples in dilutions correspond-

TABLE 1. Protective properties of mouse and human measles virus-specific serum antibodies and IgG preparations

Neutralization test ^a	Neutralization antibody titer of mouse serum ^b		Neutralization concn of mouse IgG ^c		Neutralization antibody titer of human serum ^d		
	Control	Anti-MV	Control	Anti-MV	S-1	S-2	S-3
VN ₁₀₀	<10	1,280	<800 µg/ml	25 µg/ml	80	160	640
PRNT	45–53	10,965–12,853	<800 µg/ml	2.70–3.19 µg/ml	1,016	1,265	5,511
FN ₁₀₀	<4	<4	<1.75 mg/ml	<1.75 mg/ml	<4	<4	4

^a VN₁₀₀, complete (100%) virus neutralization; PRNT, plaque reduction neutralization test (50% reduction of the virus input); FN₁₀₀, complete (100%) cell-cell fusion inhibition.

^b Neutralization antibody titer of pooled serum samples from MV-immunized (anti-MV) and control IfnarKo-CD46Ge mice.

^c Neutralization concentration of the cold-ethanol-purified IgG from MV-GFP immunized (anti-MV) and control IfnarKo-CD46Ge mice.

^d Serum samples from three MM patients were tested for neutralization antibody titer, and two of the three (S-1 and S-2) were selected for antibody enhancement experiments.

ing to 5 to 25 µg/ml purified IgG increased infection similarly (not shown). In contrast to intact IgG, Fab fragments isolated from MV-hyperimmune mouse serum could not enhance infection, indicating the importance of the Fc portion of the immunoglobulin molecule (Fig. 5C). These data point to an infectivity enhancement mechanism whereby FcγR-mediated viral attachment is followed by F-protein-mediated membrane fusion and virus entry.

Serum samples from two human subjects enhanced infection of J774A.1 cells more than 10-fold at dilutions of 1:100 and 1:1,000 that were still capable of neutralizing infectivity for Vero cells (Fig. 5D). The VN test results demonstrated that sera were highly protective, with a PRN titer of >1,000. In contrast, the fusion inhibition titer was very low (Table 1).

Antibody-dependent enhancement of infection in measles-susceptible mice. IFN type I receptor knockout (IfnarKo) is sufficient to render mice susceptible to MV infection. MV-CEA infection can be quantitated in IfnarKo mice by measuring the serum level of human CEA (31). To determine whether antibody-dependent enhancement of measles virus infection occurs in vivo, 100 µg purified IgG from MV-immunized or control animals was administered i.p. to IfnarKo mice, and three days later, 2.5×10^6 MV-CEA was administered by the same route. Serum CEA concentrations determined 4 days after viral inoculation demonstrate a significant enhancement of infection in naïve mice receiving anti-MV IgG (Table 2). CEA concentrations in mice pretreated with MV-specific IgG varied from 3,500 to 7,600 ng/ml versus 1,700 ng/ml in mice receiving control IgG or 2,700 ng/ml in the control mice (no IgG treatment).

In a subsequent experiment, we used IfnarKo mice expressing human CD46, one of the natural viral receptors. Macrophages are the primary target of MV infection in this inbred mouse line (30, 40). The mice were immunized with three doses of MV-GFP and challenged 1 week later with MV-CEA. As shown in Table 3, immunized mice were completely refractory to MV-CEA infection. On day 3 postchallenge, CEA levels were very high in nonimmune control animals but were essentially undetectable in the preimmunized animals. To demonstrate that antibody-mediated enhancement has the potential to “bypass” adaptive immunity to measles, these mice were immunized i.p. with three doses of MV-GFP and then treated with four i.p./s.c. injections of IgG purified from MV-immunized or control animals. All animals were then challenged i.p. with MV-CEA prior to determination of their se-

rum concentration of CEA 3 days later. As shown in Fig. 6A, CEA levels were high in MV-immune animals challenged with MV-specific, but not control IgG, and were approximately seven times higher than the corresponding CEA levels in non-immunized animals, indicating that IgG enhancement not only helped the virus to circumvent the preexisting anti-MV response but also resulted in more efficient infection than in an immunologically naïve host.

In parallel with CEA measurements, serum samples were assayed for enhancing, neutralizing, and immunoreactive anti-MV antibodies. All immunized mice showed strong neutralizing antibody titers as measured by the VN test and indirect immunofluorescence test (not shown). There was no apparent modulation of the antibody response among the groups that received MV-specific or control IgG, and the same pattern of reaction with MV proteins was seen by immunoblotting (Fig. 6B). All 10 sera from group 1 mice increased MV infectivity in J774A.1 cells 15- to 30-fold at a neutralizing dilution of 1:1,000 (Fig. 6C and D). However, at a dilution of 1:100, the same sera were strongly protective and did not promote infection of J774A.1 cells (Fig. 6D).

DISCUSSION

Here we have demonstrated that certain IgG antibodies recognizing MV, a representative member of the *Paramyxoviridae* family, can increase the efficiency of virus entry into cells of the monocyte lineage. This was demonstrated using murine monoclonal antibodies, polyclonal mouse IgG, and human sera. The tropism of MV is naturally restricted to primates, and cell lines of mouse origin are resistant to infection. Thus, mouse macrophages were resistant to MV infection even at high MOIs but could efficiently support the entry of IgG-opsonized virions. Since MV-Edm can enter human monocytes and macrophages through natural receptors, we used a recombinant virus ablated in their attachment sites for CD46 and SLAM (24) to study antibody-dependent enhancement on human macrophages. Antibody-enhanced infectivity was confirmed for both human monocytic lines and primary human macrophages differentiated in vitro from monocytes. Antibody concentrations mediating enhancement of MV entry in monocytes were able to block infectivity in cells of epithelial origin. In contrast to the H-specific antibody CL55, neither H-specific Fab fragments nor H-specific IgM could enhance MV entry in monocytes or macrophages, indicating involvement of a FcγR-

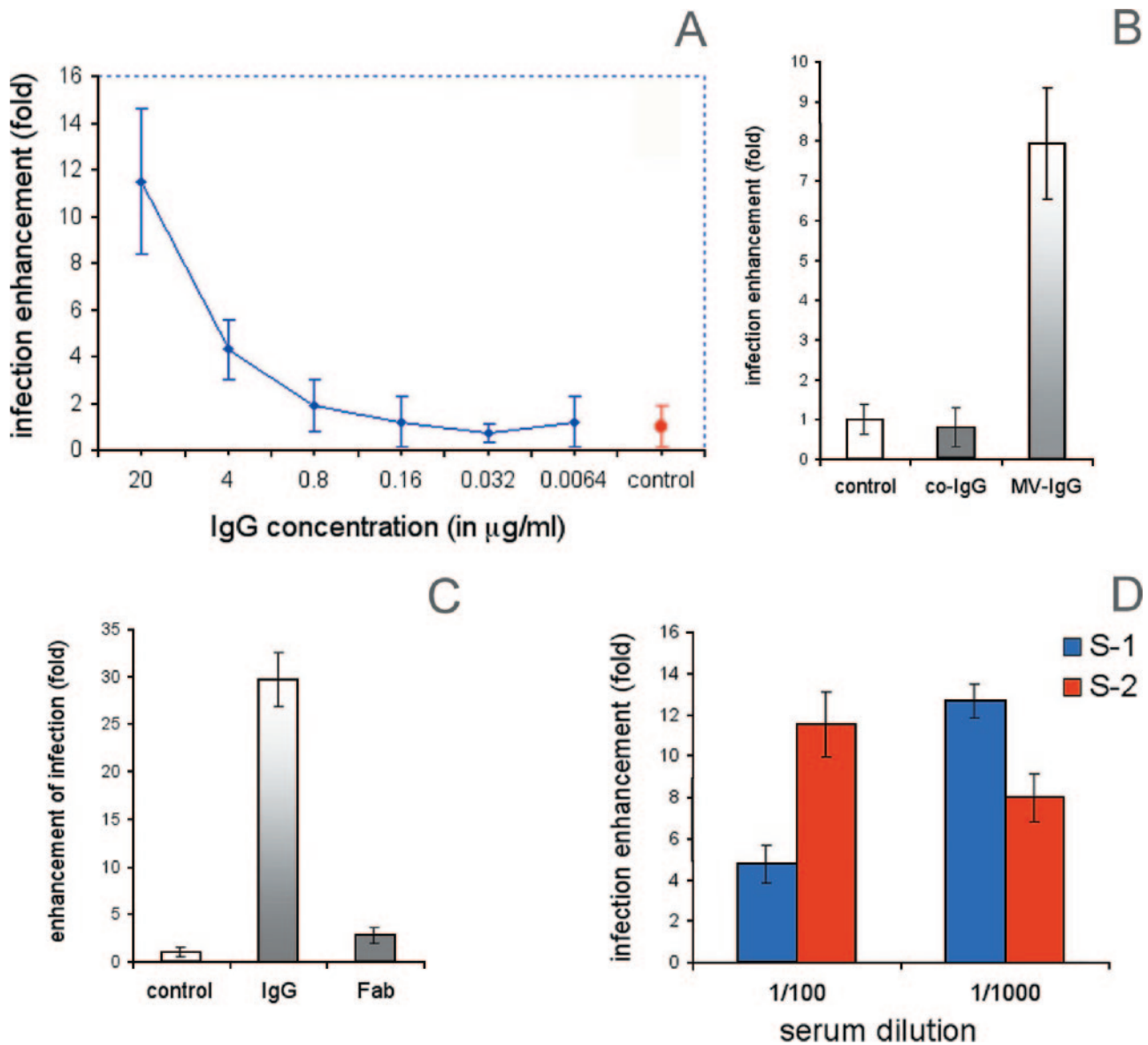


FIG. 5. Enhancement of MV infection in J774A.1 cells in the presence of mouse serum IgG and diluted human serum samples. (A) Effects of various concentrations of affinity chromatography-purified MV-specific mouse IgG on MV-GFP infection in J774A.1 macrophages compared to the control without antibody. (B) Enhancement of infection by MV-specific (MV-IgG) mouse IgG compared to control mouse IgG (co-IgG) or no IgG. Immunoglobulins were purified by cold ethanol precipitation and used at a concentration of 20 µg/ml. MV-GFP was used at a MOI of 0.1 ($P < 0.001$). (C) Purified Fab fragments could not enhance MV infectivity. IgG was purified by cold ethanol precipitation and fractionation on a Superose 6 column. Fab fragments were isolated using an ImmunoPure kit (Pierce). The results are from a representative experiment using 10 µg/ml IgG or 25 µg/ml Fab and MV-GFP at a MOI of 0.5 ($P < 0.001$). (D) MV immune human serum can enhance infection more than 10-fold at dilutions of 1:100 and 1:1,000. MV-GFP was incubated with serum samples (S-1 and S-2) for 15 min and then added to J774A.1 cells for 1 h of incubation ($P < 0.001$).

mediated mechanism. A functional F protein was required for viral internalization, because preincubation with an anti-F monoclonal antibody or FIP blocked infection. Complement cascade activation did not promote the entry of MV via CRs and inhibited anti-H IgG-mediated enhancement of MV infectivity in monocytic cell lines. MV entry into monocytes and macrophages was also enhanced by polyclonal IgG isolated from sera from measles-immune mice and by appropriately diluted sera from measles-immune mice and humans. After natural measles virus infection or immunization with live vac-

cine, antibodies blocking hemagglutination (predominately anti-H) are produced in much higher abundance than those blocking fusion (12), and this may provide an explanation for the infectivity enhancements seen with diluted human and mouse sera. Thus, at dilutions that could no longer prevent cell-cell fusion, serum IgG against the H protein could still promote infection of cells via FcγR.

Phagocytosis is a potent effector mechanism for pathogen elimination and is mediated by different cellular receptors (1, 48). Mannose receptors can directly interact with bacterial cell

TABLE 2. Human CEA level in serum samples from IfnarKo mice injected i.p. with purified IgG 3 days prior to the i.p. challenge with MV-CEA

Mouse no.	Human CEA level (ng/ml) in mice injected i.p. with ^a :		
	PBS	Control IgG	MV-specific IgG
1	2,350	1,615	7,600
2	2,625	1,670	4,540
3	3,940	1,715	3,525

^a Mice were injected with 100 µg IgG (in 500 ml PBS) isolated from nonimmunized or MV-GFP-immunized animals. IgG was purified by cold ethanol precipitation and subsequent fractionation on Superose 6 gel chromatography column. The control group received only PBS.

wall components, whereas Fc and CRs do not recognize pathogens unless they are first opsonized with antibodies or complement proteins. Three different types of FcγR are expressed on phagocytes and other immune cells (50). Binding and cross-linking of these FcγRs stimulate signal transduction followed by engulfment and destruction of bacteria and viruses in the phagosomes. However, certain intracellular pathogens, including chlamydiae, human immunodeficiency virus, and some of the deadliest filo- and flaviviruses, can use FcγR-mediated attachment as a mechanism for enhanced infectivity (14, 32, 33, 44, 45). Components of the complement cascade can also enhance viral infectivity via CRs expressed on the surfaces of macrophages (3, 39). One interesting and still unexplained observation is enhancement of fusion by antiviral antibodies in epithelial cells infected with Newcastle disease virus (16). In addition, signal transduction via FcγR can have multiple effects on monocytes and macrophages, including upregulated cytokine expression and immunomodulation (43, 48). IL-12 production by cells of monocytic lineage plays a central role in the regulation of cellular immunity as a potent inducer of the Th1 response (47). Both FcγRs and CRs are negative regulators for IL-12 (11, 18). IgG-enhanced infection of macrophages can result in suppression of host antiviral defenses by FcγR ligation triggered by the early release of IL-10 and Th2-type response switch (9). IL-10 induces expression of suppressor of cytokine signaling proteins, which inhibit alpha/beta IFN signaling in response to viral replication (43). MV infection inhibits IL-12 secretion and skews cytokine expression to induce a predominant Th2 response with increased IL-10 and IL-4 production (37). Thus, antibody-mediated enhancement of infection may also contribute to cytokine alteration and modulation of the innate immune mechanisms during measles.

Monocytes and macrophages are early targets for MV, playing a key role in disease pathogenesis and in shaping the antiviral immune response (7). Regional lymphoid tissue is the primary site for viral amplification. Infected monocytes and lymphocytes (to a lesser extent) disseminate via the bloodstream to various organs where MV can be detected in tissue macrophages and endothelial and epithelial cells. Individuals immunized with FIMV responded weakly to the F antigen, and imbalance in H- and F-specific antibody titers has been one of the widely accepted explanations for the pathogenesis of atypical measles (12, 26). Our work demonstrates that a level of anti-MV IgG that is protective for epithelial cells can, in the absence of adequate fusion-blocking antibodies, favor MV rep-

TABLE 3. Efficacy of vaccination with MV-GFP

Mouse group ^a	Challenge route ^b	Serum CEA level (ng/ml) (mean ± SD) ^c
Naive	i.v.	458 ± 286.7
Vaccinated	i.v.	<5
Naive	i.p.	7,840 ± 5,381.1
Vaccinated	i.p.	7.8 ± 3.8

^a Groups of five mice were vaccinated repeatedly with three doses of 10⁷ TCID₅₀ MV-GFP. Nonimmunized mice were used as controls.

^b Immunized animals and controls were rechallenged by 10⁷ TCID₅₀ MV-CEA i.v. or i.p.

^c On day 3 after MV-CEA injection, serum samples were diluted 1:10. The human CEA concentration was measured 3 days after rechallenge (i.p. or i.v.) with MV-CEA of the immunized IfnarKo-CD46Ge mice. The lowest detectable concentration by this test was >5 ng/ml.

lication within monocytes and macrophages, thus providing a new hypothesis for the possible pathogenesis of atypical measles. However, while our *in vivo* studies support the idea that enhancing IgG antibodies may be relevant to the pathogenesis of atypical measles, additional studies will be required to prove or disprove this question. At this time there is no murine model that recapitulates the features of atypical measles, and rhesus monkeys provide the only existing animal model that can be used to address hypotheses concerning the pathogenesis of this disease (35). Nevertheless, our *in vivo* mouse data are provocative, because they demonstrate that *in vivo* conditions exist wherein the entry of measles virus into cells of the monocyte/macrophage lineage can be substantially enhanced by anti-MV antibodies, despite the presence of fusion-blocking antibodies and complement proteins. It should be stressed that for these studies we employed an i.p. route of virus administration, thereby giving the virus direct access to the peritoneal macrophages. Administration of the virus by nasal instillation to mimic the natural route of infection or directly into the bloodstream, the preferred route for an oncolytic virus, might have a different outcome due to differences in the relative concentrations of neutralizing and enhancing antibodies and of host complement proteins in different body compartments.

Attenuated MV is a promising candidate for oncolytic virotherapy. However, in measles-immune subjects, it is predicted that MV administered i.v. will be rapidly inactivated by preexisting antibodies and complement. Isolated PBMCs could be inoculated *in vitro* and then reinjected i.v. in the same patient, enabling MV to escape from antibody neutralization in the bloodstream, mimicking cell-associated viremia during natural measles (7, 12). In the presence of H-protein-specific IgG, PBMCs can even be infected with MVs whose natural tropisms have been ablated, offering a new strategy for intravenous delivery of fully retargeted oncolytic MVs using cell carriers infected via FcγRs.

In conclusion, we have shown for the first time that morbillivirus infectivity is subject to antibody-mediated enhancement via FcγRs expressed in monocytes and macrophages and that this works efficiently *in vivo*, allowing MV to overcome a strong preexisting humoral immune response. Our results provide new evidence suggesting a previously unidentified mechanism contributing to morbillivirus pathogenesis in which H-specific IgG antibodies might promote the spread of measles virus

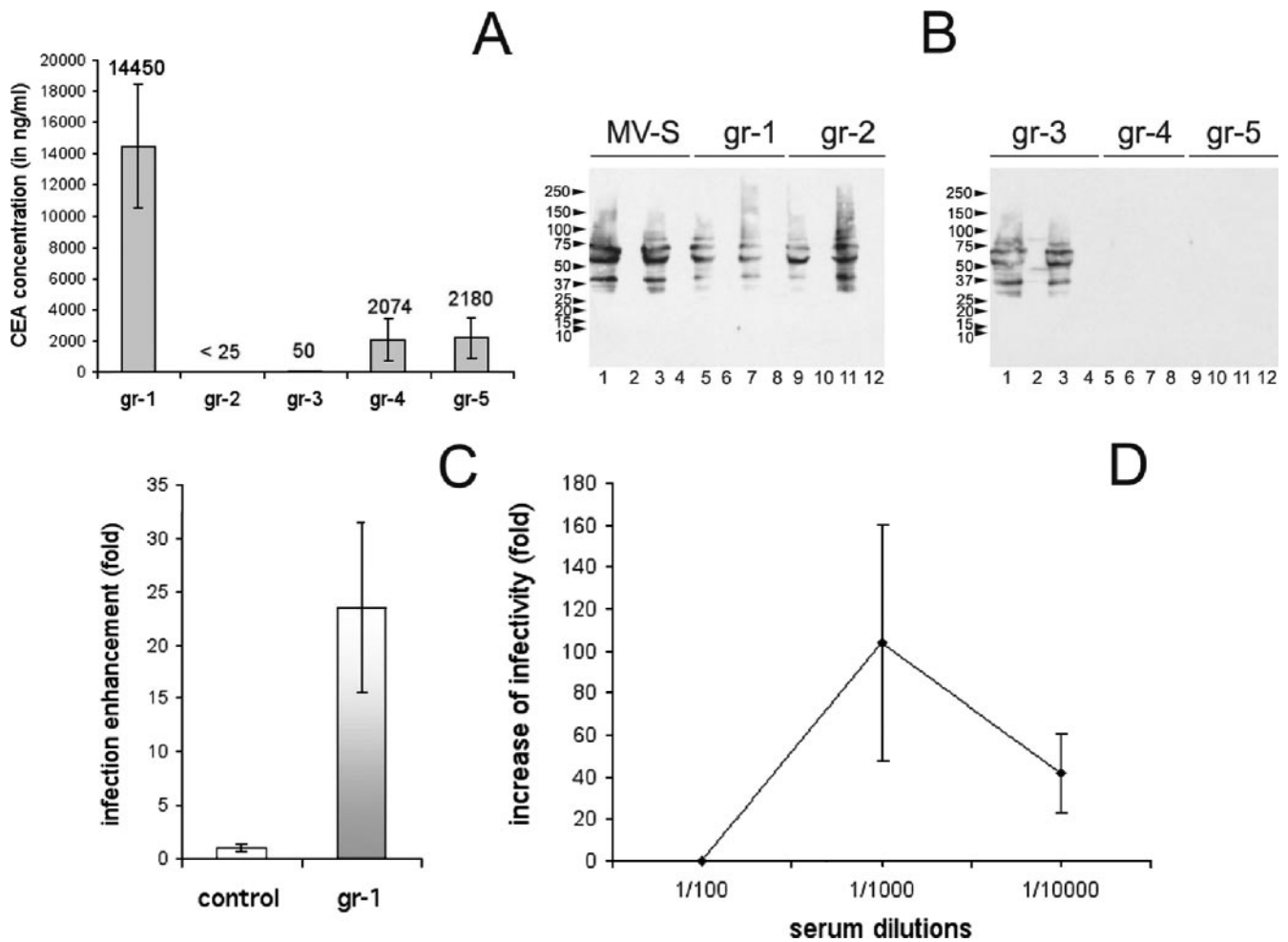


FIG. 6. In vivo enhancement of MV infection by MV-specific IgG in transgenic *IfnarKo-CD46Ge* mice. (A) Effects of repeated injections of MV-specific IgG on MV infectivity in vaccinated mice. Groups 1, 2, and 3 (gr-1, gr-2, and gr-3) were immunized three times with 2×10^6 TCID₅₀ MV-GFP. Groups 4 and 5 were nonimmunized controls. One week after the last vaccination, group 1 was injected with affinity chromatography-purified MV-specific IgG. Groups 2 and 4 were injected with the same amount of control IgG. Mice from groups 3 and 5 received only PBS. Three days after the last IgG injection, all animals were challenged i.p. with 2×10^6 TCID₅₀ of MV-CEA, and 72 h later, serum CEA level was determined. The results (mean \pm SD) are calculated from the CEA values (in nanograms per milliliter) measured twice for each sample ($P < 0.001$). (B) Anti-MV antibodies in sera from measles-immunized mice from the experiment described above. MV-GFP lysates were loaded in lanes 1, 3, 5, 7, 9, and 11; control Vero cell lysates were loaded in lanes 2, 4, 6, 8, 10, and 12. Gels were transferred to nitrocellulose membranes and immunoblotted with representative mouse sera (two mice per group) collected 3 days after MV-CEA challenge. The positions of the marker protein standards (in kilodaltons) are indicated by black arrowheads to the left of the gels. MV-S, control MV-specific mouse serum pooled from 20 MV-GFP-immunized control mice. (C) Enhancement of MV infection of J774A.1 macrophages by sera from group 1 (gr-1) mice or control measles-immunized mice. Sera were diluted 1:1,000 and incubated for 15 min with MV-GFP prior to inoculation of the cells at a MOI of 0.1 for 45 min. The samples were run in six wells of 24-well plates, and the number of GFP-expressing cells was counted 3 days after infection ($P < 0.001$). (D) Enhancement of MV-GFP infectivity on J774A.1 cells by serum dilutions 1:100, 1:1,000, and 1:10⁴ of group 1 mice. Virus was incubated with serum for 30 min, and cells were inoculated for 30 min. Infected cells were counted on day 3 after infection.

infection among Fc receptor-expressing host cells. This may have been of major importance in the pathogenesis of atypical measles in measles-immune subjects who were immunized with FIMV, since these patients may have had a suboptimal anti-F response. Our data show that F-protein-specific antibodies are inhibitory to the antibody-dependent infectivity enhancement, an observation that may have major significance for the design of recombinant paramyxovirus vaccines, suggesting that they should not be focused exclusively on the H glycoprotein. Finally, our results suggest a novel strategy for delivery of fully retargeted (cancer-specific) oncolytic viruses to disseminated metastases in cancer patients whereby Fc γ R-expressing cell

carriers can be infected with IgG-opsonized virus and subsequently deliver virus progeny to tumor cells by heterofusion at the target site.

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