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Baculovirus Expression of the Nucleoprotein Gene of Measles Virus and Utility of the Recombinant Protein in Diagnostic Enzyme Immunoassays

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A recombinant baculovirus that expresses the nucleoprotein gene of measles virus (Edmonston vaccine strain) under the transcriptional control of the polyhedrin promoter was generated. The expressed protein (B-MVN) comigrated with the authentic viral nucleoprotein as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it was phosphorylated. The B-MVN protein proved to be reactive with monoclonal antibodies in radioimmunoprecipitations, and it was immunogenic, eliciting in mice antisera that recognized the native nucleoprotein. In addition, the B-MVN protein was evaluated as a replacement source of antigen for whole virus in enzyme immunoassays (EIAs) for detection of measles virus-specific immunoglobulin M (IgM) and IgG antibodies. A capture IgM EIA with the B-MVN protein as antigen detected specific IgM antibodies in 18 (72%) acute- and all convalescent-phase specimens from 25 clinical measles cases and exceeded 99% specificity with 120 control specimens. An indirect IgG EIA with the B-MVN protein detected specific IgG antibodies in 129 of 131 (98%) serum specimens with antibodies to measles virus, and results obtained from testing 268 additional serum specimens were better correlated with measles virus-neutralizing antibodies than those obtained with a commercial EIA.

Measles continues to be a major problem in developing countries, with an estimated 49 million cases reported in 1989, resulting in the death of 1.5 million children worldwide (20). Within the United States, eradication of measles remains elusive despite the implementation of vaccine regimens since 1963. As a result of vaccine failure or nonvaccination, more than 27,000 measles cases were reported in 1990, 50% more than the number in 1989 and nearly 10 times that reported in 1988 (7). Coincident with this resurgence of measles, and owing to the complications which often occur with infection, has been a renewed interest in measles virus diagnostic serology. Among available methods, enzyme immunoassays (EIAs) are widely used because of their high sensitivity and inherent practicality. However, most current commercial and research EIAs rely on measles virus-infected cells as antigen, which can be difficult to reproduce in high titer.

In an effort to produce a standard, high-titer assay reagent, we constructed a recombinant measles virus nucleoprotein by using a baculovirus expression system. Recombinant baculovirus vectors, which conserve the 5′ sequence immediately upstream of the initiation codon of the polyhedrin gene, are well known for their ability to express large quantities of foreign protein under the transcriptional control of the polyhedrin promoter (14). The nucleoprotein appeared to be an excellent candidate for expression, as it is the dominating internal structural protein of the measles virus virion and has been identified as a major target of the human T-cell response important for viral clearance (4). In this study, we characterized the immunogenic properties of the baculovirus-expressed measles virus nucleoprotein, B-MVN protein, and evaluated its efficacy as an antigen in EIAs for the detection of specific immunoglobulin M (IgM) and IgG antibodies to measles virus.

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MATERIALS AND METHODS

Cells and virus. The Edmonston vaccine strain of measles virus was obtained from the American Type Culture Collection (Rockville, Md.); it was deposited by J. Enders after 24 passages in human kidney cells and 28 passages in human amnion cells. CV-1 cells (African green monkey kidney) were cultivated in Dulbecco's modified medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics and were used to support virus growth.

Spodoptera frugiperda (Sf9) cells and *Autographa californica* nuclear polyhedrosis virus were obtained from M. D. Summers (Department of Entomology, Texas A&M University, College Station, Tex.). Sf9 cells were cultured in supplemented Grace's insect medium (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum, 0.55% bovine serum albumin (Boehringer Mannheim, Indianapolis, Ind.), gentamicin sulfate, and amphotericin B (Fungizone) as additives.

Recombinant virus construction and purification. Transfer vector pAcYM1 (14), obtained from D. H. L. Bishop (NERC Institute of Virology, Oxford, United Kingdom), was modified by the insertion of a multiple cloning region at the original *Bam*HI site to produce pAcYM1B/S and was donated by A. Sanchez (Centers for Disease Control, Atlanta, Ga.). Plasmid constructs were confirmed by restriction endonuclease and DNA dideoxy sequence analyses as described by Maniatis et al. (13). Transfection of *A. californica* nuclear polyhedrosis virus and vector DNA into cultured Sf9 cells was achieved through use of a calcium phosphate precipitation technique as described by Summers and Smith (18). Dot blot analyses for isolation of a recombinant virus were performed on GeneScreen hybridization transfer mem-

branes (New England Nuclear, Boston, Mass.) by using ^{32}P-labeled nick-translated cDNA probes.

MAbs. Monoclonal antibodies (MAbs) 83VIIKK2 and 81I168, specific for the measles virus nucleoprotein (5), were purified from mouse ascitic fluid and used for immunoprecipitation and immunofluorescence studies. These MAbs and MAb 79XVV17, specific for the measles virus hemagglutinin protein, were biotinylated as previously described (2) for use as detector reagents in capture IgM EIAs.

Protein analyses. Sf9 or CV-1 cells infected at a multiplicity of infection of 1 with the recombinant baculovirus or with plaque-purified Edmonston virus, respectively, were lysed in Laemmli sample buffer (12), and total cellular proteins were resolved by electrophoresis through 4 to 20% Mini-PROTEAN gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad, Melville, N.Y.). Following electrophoresis, the recombinant nucleoprotein was visualized both by staining with Coomassie brilliant blue and by Western blotting (immunoblotting) with MAbs specific for the nucleoprotein at a 1:50 dilution and a protein G-gold conjugate (Bio-Rad).

For radioimmunoprecipitations, infected cell monolayers were starved in medium without methionine or phosphate prior to the addition of [^{35}S]methionine (50 μCi/ml) or $^{32}P_i$ (50 μCi/ml) for 1 h. Measles virus-specific proteins were immunoprecipitated by using MAbs specific for the nucleoprotein at a 1:20 dilution and Gammabind protein G (Genex, Gaithersburg, Md.) before SDS-PAGE separation and fluorographic enhancement (Amplify; Amersham, Arlington Heights, Ill.). Immunoprecipitations were also similarly performed with a 1:20 dilution of polyclonal antisera generated in mice. BALB/c mice were inoculated intraperitoneally with 0.5 ml of crude lysate from recombinant baculovirus-infected cells and were bled 1 and 2 weeks postimmunization.

Immunofluorescence. Recombinant baculovirus protein expression was assayed in acetone-fixed Sf9 cells by indirect immunofluorescence (19) with a 1:30 dilution of MAbs specific for the nucleoprotein and a fluorescein isothiocyanate-conjugated secondary antibody.

Serum specimens. Human serum specimens used in evaluation of the recombinant EIAs were obtained from Centers for Disease Control specimen collections or were available from outside sources. Test panels comprising a total of 533 specimens obtained from 458 persons included (i) 25 acute- and convalescent-phase specimen pairs from clinical measles cases collected within approximately 2 days and 2 to 3 weeks after onset of rash, respectively; (ii) 120 total specimens from 40 healthy adult blood donors, 18 healthy children, and 37 children and adults with clinical and serological evidence of recent infection with other viruses, including 10 with respiratory syncytial virus, 7 with rubella virus, and 5 each with parainfluenza virus types 1 and 3, mumps virus, and human parvovirus; (iii) 160 total specimens from the 40 blood donors listed above, convalescent-phase specimens from the 25 clinical measles cases listed above, and pre- and/or postvaccination specimens from 70 age-appropriately vaccinated children; and (iv) 268 specimens from as many health care workers, donated by K. MacDonald (Minnesota Department of Health, Minneapolis, Minn.) and screened for plaque reduction neutralizing antibodies (1, 16) by P. Albrecht (Food and Drug Administration, Bethesda, Md.).

Antigen for EIAs. B-MVN antigen was prepared from Sf9 cells infected with the recombinant baculovirus at a multiplicity of infection of 1 and harvested 72 h postinfection. Monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.2, to remove residual fetal bovine serum, and the cells were resuspended in PBS to a final concentration of 5×10^6 cells per ml. The cell suspension was then subjected to freeze-thaw three times, and large cellular debris was pelleted by low-speed centrifugation ($500 \times g$ for 15 min). The supernatant was then collected and stored at −40°C until use. The Edmonston vaccine strain of measles virus grown in E6 Vero cells was used for preparation of antigen for use in whole virus EIAs as previously described (9). Uninfected Sf9 and E6 Vero cells were processed similarly for use as negative control antigen.

EIAs. B-MVN EIAs were adapted from whole virus assays previously described (9). Assays were modified to accommodate comparisons between whole virus and recombinant protein antigens.

Capture IgM EIAs. Goat anti-human IgM antibodies diluted 1:1,000 in 0.01 M PBS were coated onto microtiter plates for 1 h at 37°C. Plates were washed, and serum specimens, diluted 1:100 in PBS with 0.5% gelatin and 0.15% Tween 20 (PBS-G-T), were each added to four consecutive wells and incubated for 1 h at 37°C. Plates were washed, and B-MVN antigen and Sf9 negative control antigen, diluted 1:250 each in PBS-G-T with 4% normal goat serum and 0.3% sodium deoxycholate, or whole measles virus antigen and E6 Vero negative control antigen, diluted 1:10 each in PBS-G-T, were added to duplicate wells for each specimen and incubated for 1 h at 37°C. Plates were washed, and a 1:3,000 dilution of biotinylated MAb 83VIIKK2 in PBS-G-T or a 1:4,000 dilution of biotinylated MAbs 81I168 and 79XVV17 in PBS-G-T was added to wells containing recombinant or whole virus antigen, respectively, and incubated for 1 h at 37°C. Plates were washed, and a 1:3,000 dilution of streptavidin-peroxidase in PBS-G-T was added to all wells and incubated for 20 min at 37°C. After a final wash, a 3,3′,5,5′-tetramethylbenzidine substrate solution was added and incubated for 15 min at room temperature. The reaction was then stopped by acidification, and the color intensity was determined photometrically.

Indirect IgG EIAs. B-MVN antigen and Sf9 negative control antigen, diluted 1:1,000 each in PBS, or whole virus antigen and E6 Vero negative control antigen, diluted 1:40 each, were added to duplicate wells of a microtiter plate and incubated for 1.5 h at 37°C. Plates were washed, and serum specimens, diluted 1:100 in PBS-G-T with 4% normal goat serum and 4% of the appropriate negative control antigen, were added and incubated for 1 h at 37°C. Plates were washed, and a 1:3,000 dilution of goat anti-human IgG peroxidase conjugate in PBS-G-T was added to all wells and incubated for 1 h at 37°C. After a final wash, the substrate solution was added, and color development was as previously described.

EIA results were expressed as net absorbance or P − N values, defined as the average difference in measured absorbance values between duplicate wells of positive (P) and negative control (N) antigen for each serum specimen. The cutoff value for a positive test was taken as the mean P − N value plus three standard deviations for a panel of 20 control specimens with no detectable plaque-neutralizing antibodies. Cutoffs chosen for the capture IgM and indirect IgG assays using the B-MVN protein and whole virus antigens were P − N = 0.12 and 0.11 and P − N = 0.09 and 0.21, respectively. P/N ratios of ≥3 were also required to account for occasional specimens with high background signal.

Commercial EIA. The MEASELISA II test kit (Whittaker Bioproducts, Inc., Walkersville, Md.) was chosen for com-

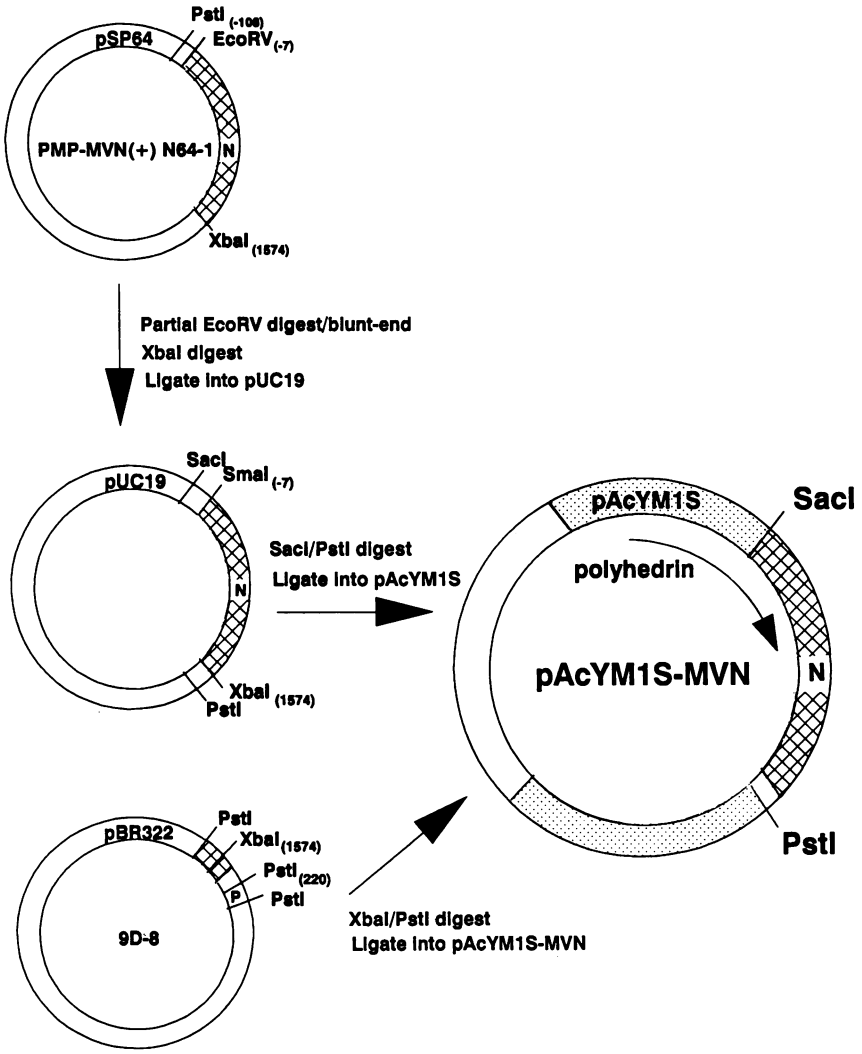

FIG. 1. Schematic diagram of the subcloning strategy for the insertion of the full-length nucleoprotein gene of measles virus into the baculovirus transfer vector pAcYM1S. Restriction endonuclease digests performed are as indicated. Hatched or stippled areas indicate coding regions of the nucleoprotein or the polyhedrin gene, respectively.

parison with the B-MVN IgG EIA, and results were determined according to the manufacturer's instructions.

RESULTS

Recombinant virus construction and purification. Two cDNA clones encoding the nucleoprotein gene of measles virus (17) were ligated to generate a full-length open reading frame suitable for insertion into baculovirus expression vector pAcYM1S (Fig. 1). The resultant plasmid, pAcYM1S-MVN, contained a 1.855-kb insert originating 7 bp upstream from the first in-frame AUG of the *N* gene and extending through the stop codon, intergenic region, and the first 200 nucleotides of the *P* gene. A recombinant baculovirus was isolated after one round of limiting dilution cloning based on selection using dot blot hybridizations to the *N* gene. Successive rounds of plaque purification were confirmed by indirect immunofluorescence on infected Sf9 cells by using mouse MAbs to the nucleoprotein. The purified recombinant baculovirus yielded a high titer stock of 2×10^8 PFU/ml and displayed no evidence of occluded virus in infected cell nuclei or of the polyhedrin protein by SDS-PAGE.

Protein analyses. Total cellular proteins derived from Sf9 cells infected with the recombinant baculovirus were resolved by SDS-PAGE. Detection by means of Coomassie brilliant blue staining and with antibody probes in Western blotting (immunoblotting) revealed that the B-MVN protein migrated with an apparent molecular mass of 58 kDa (data not shown). No such protein was detected in lysates representative of uninfected or wild-type baculovirus-infected cells. Infected cell monolayers were also radiolabeled, and measles virus-specific proteins were immunoprecipitated with MAbs specific for the nucleoprotein (Fig. 2). The B-MVN protein appeared to comigrate with the full-length viral nucleoprotein, and it was phosphorylated. Additionally, antisera generated in mice against the B-MVN protein were capable of precipitating the authentic nucleoprotein. In all cases, uninfected and wild-type baculovirus-infected cell lysates did not precipitate any measles virus-specific proteins (data not shown).

Several faster-migrating species of the recombinant nucle-

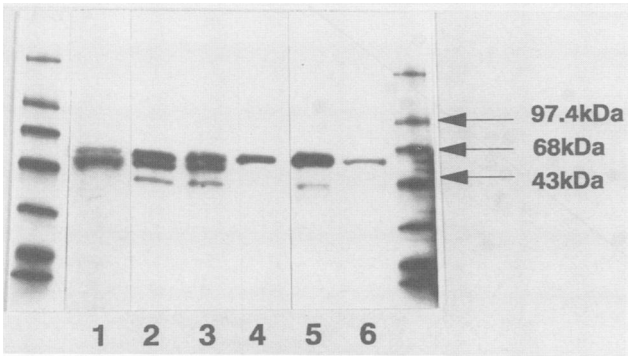

FIG. 2. A composite autoradiogram of infected cell lysates radiolabeled and immunoprecipitated with MAbs specific for the nucleoprotein before resolution by 4 to 20% gradient SDS-PAGE and fluorography. Lanes: 1, recombinant baculovirus-infected Sf9 cell lysate radiolabeled with $^{32}P_i$ and immunoprecipitated with MAbs 81I168 and 83VIIKK2; 2 and 3, recombinant baculovirus-infected Sf9 cell lysate radiolabeled with [^{35}S]methionine and immunoprecipitated with MAbs 81I168 and 83VIIKK2, respectively; 4 and 5, Edmonston virus-infected CV-1 cell lysate radiolabeled with [^{35}S]methionine and immunoprecipitated with 81I168 and 83VIIKK2 at 5 and 20 h of exposure, respectively; 6, Edmonston virus-infected CV-1 cell lysate radiolabeled with [^{35}S]methionine and immunoprecipitated with antisera generated in mice.

oprotein, including a characteristic 45-kDa form which has been previously described for measles virus-infected cells (5), were also detected in radioimmunoprecipitations (Fig. 2). Such truncated proteins may result from proteolytic cleavage events or could represent initiation at a second in-frame AUG. Upon infection with the recombinant baculovirus at a multiplicity of infection of 10, however, the 45-kDa form and an additional protein with an apparent molecular mass less than 15 kDa predominated. It is interesting that upon further analysis, the 45- and 15-kDa proteins had lost reactivity with MAbs 81I168 and 83VIIKK2, respectively (data not shown).

Detection of specific IgM antibodies. The specificities of MAbs 81I168 and 83VIIKK2 for the B-MVN protein were determined by titrating each MAb against the recombinant protein adsorbed directly onto a microtiter plate (Fig. 3A). Titers for both MAbs exceeded 1:16,000 from a starting total protein concentration of 1 mg/ml. The reactivity of each MAb to the B-MVN protein bound to an unlabeled MAb on the solid phase was evaluated for efficacy in a capture assay format. Whereas 81I168 and 83VIIKK2 gave comparable results as detector antibodies with unlabeled 83VIIKK2 used in the capture phase, neither reacted with 81I168 in the capture phase (Fig. 3B). Only 83VIIKK2 gave a strong signal with known IgM-positive serum samples (Fig. 3C) and was therefore used as detector antibody in the capture IgM EIA.

The sensitivities of capture IgM EIAs with the B-MVN protein and whole measles virus were compared by using acute- and convalescent-phase serum pairs from 25 persons with clinical and serological evidence of recent measles virus infection as determined by a fourfold or higher increase in complement fixation and hemagglutination inhibition titers and specific IgG antibodies by EIA. Specific IgM antibodies were detected by the B-MVN protein EIA in 18 (72%) acute-phase specimens, whereas 22 (88%), including the 18 specimens positive by the recombinant EIA, were positive with the whole virus EIA (Fig. 4). All convalescent-phase specimens were positive for IgM antibodies by both assays.

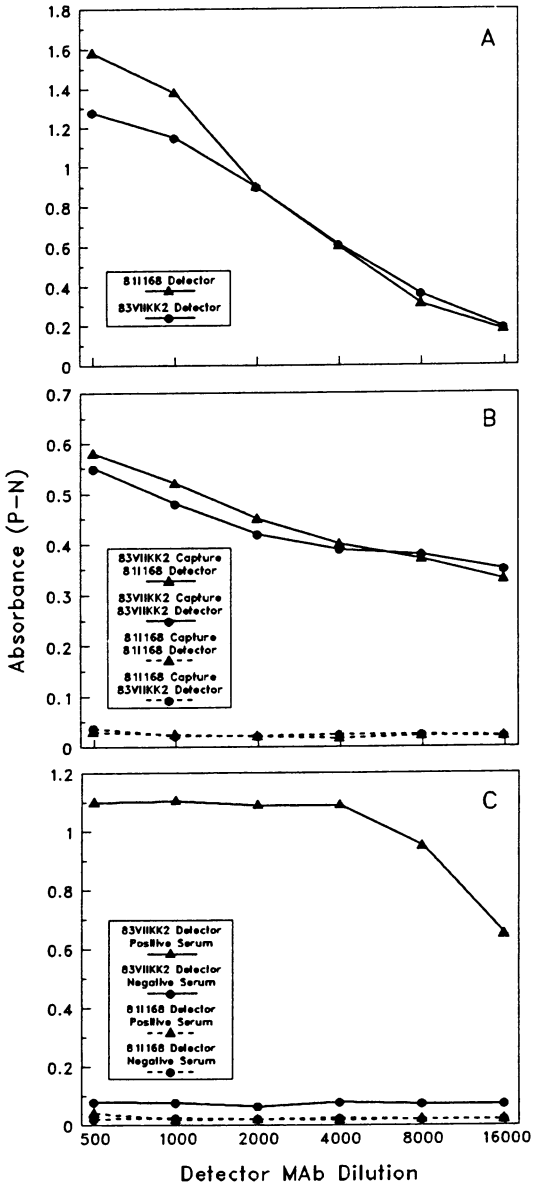

FIG. 3. Evaluation of MAbs 81I168 and 83VIIKK2 for use as detector antibodies in the capture IgM EIA. (A) Reactivities of biotinylated MAbs to the B-MVN protein fixed directly to a microtiter plate; (B) abilities of different combinations of purified capture and biotinylated detector MAbs to simultaneously bind and detect B-MVN protein; (C) abilities of biotinylated detector MAbs to identify known IgM-positive and -negative serum specimens.

The specificity of the B-MVN protein capture EIA was determined by testing 132 serum specimens from healthy persons and persons with other virus infections. Of these, one healthy person with an unknown vaccine history had detectable IgM antibodies by both the B-MVN protein and whole virus EIAs.

Detection of specific IgG antibodies. Indirect IgG EIAs with the B-MVN protein and whole measles virus as antigens were compared by using 160 serum specimens from persons with varied histories of exposure to measles virus or vaccine (Fig. 5). Specific IgG antibodies were detected by the B-MVN protein EIA in 129 of 131 (98%) specimens positive

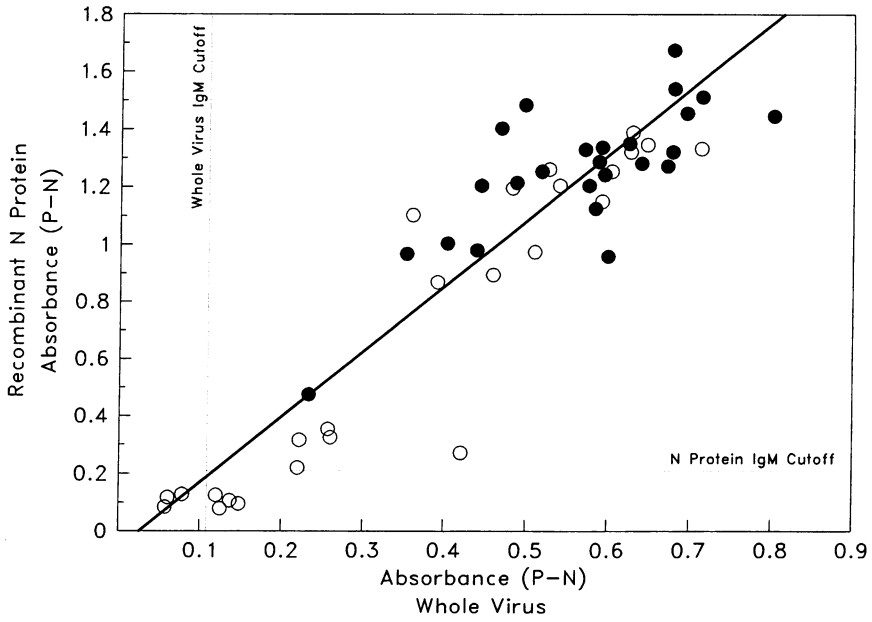

FIG. 4. Comparison of B-MVN protein and whole measles virus capture IgM EIAs with acute-phase (○) and convalescent-phase (●) serum pairs from 25 persons with clinical measles virus infection. The line is fitted by linear regression (R^2 = 0.85).

by the whole virus EIA. Two discrepant specimens obtained from healthy blood donors with unknown vaccine history were weakly positive by whole virus EIA and consistently negative by recombinant EIA. All 27 specimens without detectable IgG antibodies by whole virus EIA were also negative by the B-MVN protein EIA.

The B-MVN protein EIA was then compared with a popular commercial EIA by using 268 serum specimens from persons previously tested for plaque reduction neutralizing antibodies. A summary of the results is presented in Table 1. The B-MVN protein EIA values were generally in better agreement with detection of neutralizing antibodies than were values obtained by the commercial EIA, particularly at lower levels of neutralizing antibodies. Indeed, 48 of 109 (44%) specimens with neutralizing antibody titers of ≤120 were positive by the B-MVN protein EIA, compared with only 11 (10%) by commercial EIA. Overall, 137 of 223 (61%) specimens positive for neutralizing antibodies were positive by the B-MVN protein EIA, whereas 88 (40%) were positive by commercial EIA. Of the 45 specimens without detectable neutralizing antibodies, 1 (2%) specimen was positive by each assay.

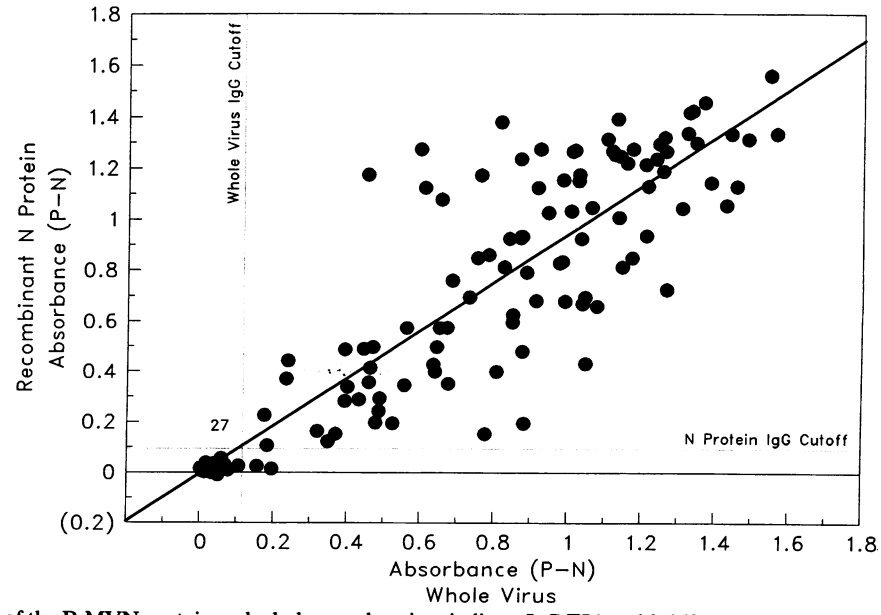

FIG. 5. Comparison of the B-MVN protein and whole measles virus indirect IgG EIAs with 160 serum specimens from persons with varied histories of exposure to measles virus or vaccine. The line is fitted by linear regression (R^2 = 0.80).

TABLE 1. Comparison of the B-MVN protein and commercial EIAs for determination of measles virus antibody status of 268 serum specimens containing various levels of plaque neutralizing antibodies

Neutralizing antibody titer[a]	No. tested	Results (%) of B-MVN protein EIA/commercial EIA[b]				Total +[c] results (%) of:	
		+/+	+/−	−/+	−/−	B-MVN protein EIA	Commercial EIA
<8	45	0 (0)	1 (2)	1 (2)	43 (96)	1 (2)	1 (2)
8–60	50	4 (8)	14 (28)	1 (2)	31 (62)	18 (36)	5 (10)
61–120	59	5 (8)	25 (42)	1 (2)	28 (47)	30 (50)	6 (7)
121–240	42	10 (24)	13 (31)	0 (0)	19 (45)	23 (55)	10 (24)
>240	72	63 (88)	3 (4)	4 (6)	2 (3)	66 (92)	67 (94)

[a] Plaque reduction neutralization antibody titers determined by Paul Albrecht, Food and Drug Administration.
[b] Specimens tested by the B-MVN protein and commercial (MEASELISA II test kit, Whittaker Bioproducts, Inc.) IgG EIAs. Results are reported as either positive (+) or negative (−) for measles virus-specific IgG antibodies.
[c] +, positive.

DISCUSSION

A recombinant baculovirus containing the measles virus nucleoprotein gene was shown to induce the synthesis of a 58-kDa phosphorylated protein that was similar to the authentic viral protein in electrophoretic mobility and in reactivity with MAbs in immunofluorescent and immunoprecipitation assays. A depletion in the amount of full-length nucleoprotein observed with infectivity of the recombinant baculovirus at a higher multiplicity of infection might be explained by an increase in proteolysis owing to a greater cell death, although the consistency of the 48- and 15-kDa products may suggest otherwise. Interestingly, upon trypsin cleavage of the viral nucleoprotein purified from ribonucleoparticles, an N-terminal 48-kDa polypeptide, which remains attached to the genome, and a 12-kDa C-terminal polypeptide result (10), and both can be distinguished by epitopic variances (6). Perhaps the B-MVN protein at high cellular concentrations mimics such proteolytic cleavage activity, although such speculation warrants further investigation. In addition, purification of the expressed protein should prove useful in fundamental research concerning structure-function studies related to transcription, replication, and encapsidation. Previous research has shown that purified nucleoprotein from Sendai virus is necessary and sufficient for in vitro genome RNA synthesis and encapsidation from preinitiated intracellular Sendai virus defective interfering particle nucleocapsid templates (3).

The expressed protein was also evaluated as a potential source of antigen for diagnostic EIAs because antibodies to the nucleoprotein appear early on and predominate during the course of infection (11, 15). The B-MVN protein could supply an abundant source of antigen necessary for the evaluation of seroconversion and seroprevalence rates within vaccinated populations and provide support for the investigation of measles virus outbreaks, eliminating dependence on cultured measles virus in diagnostic tests.

In this study, a capture IgM EIA with the recombinant antigen detected specific IgM antibodies in 72% of acute-phase specimens from clinical measles cases and exceeded 99% specificity with a broad range of control specimens. A somewhat lower sensitivity observed with the acute-phase specimens with the whole virus EIA may suggest that the specific IgM response to the B-MVN protein is delayed relative to a combined IgM response to the whole virus.

An indirect IgG EIA with the recombinant antigen detected specific antibodies in most serum specimens positive with whole virus antigen, and results obtained with a second serum panel were better correlated with the presence of measles virus-specific neutralizing antibodies than those obtained with a commercial EIA, particularly at lower antibody titers. Replacing whole virus antigen with B-MVN protein in diagnostic EIAs could theoretically cause false-negative results with specimens containing protective antibodies but with no antibodies to the measles virus nucleoprotein. This appears to be a minor problem, however, as fewer than 2% of specimens positive by the whole virus EIA were negative with the recombinant protein. Chen et al. (8) recently reported that neutralizing antibody titers of ≤120 may not provide sufficient protection to prevent development of clinical measles, even though the B-MVN protein EIA as described here could detect antibodies at this level. Increasing the cutoff value or decreasing the level of antibody detection of the B-MVN protein EIA could reduce the number of positives in this critical range, but at a loss in overall assay sensitivity. Therefore, at present, we recommend that the B-MVN protein EIA be used to report prior exposure without implying protection.

Although the plaque reduction neutralization assay provides the best estimate of protection from measles virus infection, it is unsuitable for routine use, whereas EIAs provide both a sensitive and practical method for measles diagnostics. With the availability of a recombinant nucleoprotein for use as assay antigen, standardized antibody EIAs for measles virus are now within reach.

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