Western Blot Analyses of Measles Virus Antibody in Normal Persons and in Patients with Multiple Sclerosis, Subacute Sclerosing Panencephalitis, or Atypical Measles

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A version of the Western blot was developed to detect serum antibodies against measles virus polypeptides. With this technique, a seroepidemiological survey of antibodies to the several measles virus proteins in diverse measles-related conditions was conducted. The sera were obtained from individuals with a recent or long-past history of natural measles, from persons with a history of immunization with live attenuated measles vaccine, and from patients with multiple sclerosis, subacute sclerosing panencephalitis, or atypical measles. The findings indicated that live attenuated measles vaccine elicits an antibody response qualitatively resembling that of a natural infection. In addition, multiple sclerosis patients made less antibody to the measles virus M protein than did individuals with a long-past history of natural measles. Thus, the immunological reaction of multiple sclerosis patients to measles virus is qualitatively, as well as quantitatively, different from that of normal persons. Finally, persons with subacute sclerosing panencephalitis and atypical measles mounted abnormally high antibody responses to measles virus polypeptides, in particular the P protein.

Although the incidence of clinical measles in the United States has declined to low levels since the implementation of a large-scale vaccination program (17), several questions remain regarding the pathogenesis of the measles-related syndromes of multiple sclerosis (MS), subacute sclerosing panencephalitis (SSPE), and atypical measles (AM). Although there can be little doubt that measles virus (MV) is an important etiological factor in SSPE (22) and AM (19, 22), the association with MS is based primarily on observations of slightly elevated MV serum antibody titers (2, 14) and nonspecific epidemiological considerations (3, 10, 11, 33). Attempts have been made previously to identify quantitative differences in the polypeptide specificities of the MV antibodies in MS patients (16, 25, 26, 30, 35). However, because of variations in experimental design or technique or both, these studies have yielded dissimilar results.

Many of these MV polypeptide studies have relied on the immunoprecipitation test (16, 26, 29, 35). The labor-intensiveness of this technique has limited sample sizes, and numbers have seldom been adequate to determine the statistical significance of qualitative differences in MV antibodies. To overcome this problem, a new assay was adopted. In the Western blot technique (32), denatured MV proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper, which was then cut into replicate strips. Tests of reproducibility could be performed by exposing a strip to human serum and identifying bound antibody with peroxidase-conjugated anti-human immunoglobulin G. In the analyses reported here, antibodies against only four (H, P, N, and M) of the five major MV polypeptides were evaluated. F protein antibody was not analyzed because of difficulty in resolving

this protein from breakdown products of the larger MV proteins on the polyacrylamide gel.

MATERIALS AND METHODS

Preparation of MV. A plaque-cloned preparation of the Edmonston strain of MV (infectivity titer, $10^{7.5}$ PFU/ml) was used to inoculate Vero cells at a multiplicity of infection of 10. Following the 2-h absorption period, infected cells were maintained at 37°C in serum-free Dulbecco modified Eagle medium supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml). At 24 h postinoculation, the medium was changed. When cytopathic effects involved 80 to 100% of the cells, that is, at approximately 72 h postinoculation, the infected tissue culture fluid was removed and clarified first by centrifugation at $1,000 \times g$ for 30 min and then by filtration through a 0.45 µm filter. The clarified fluid was centrifuged in a Beckman SW27.1 rotor for 2 h ($73,000 \times g$, 4°C) onto a 60% (wt/vol) sucrose cushion in TE buffer (0.005 M Tris hydrochloride [pH 7.4], 0.001 M EDTA). The resulting band on the cushion had a protein content of 770 µg/ml. This band was removed and used as the assay antigen. It was characterized by electrophoresis in a 10% polyacrylamide gel (30 mA, 3.5 h). The gel was silver stained by the method of Merrill et al. (20). Prominent bands appeared consistently at molecular masses of 79, 72, 60, and 37 kilodaltons, corresponding to the H, P, N, and M proteins, respectively, as determined by comparison with known molecular mass standards. A prominent band also appeared at the 41-kilodalton position of the F protein, but its intensity fluctuated widely in different gels. This instability may have been due to the presence of variable breakdown fragments of larger proteins, especially the N protein (28). As a result, analysis of antibody to the F protein was not attempted.

Negative controls. A flask of confluent Vero cells was mock inoculated and treated in the manner described for the assay antigen. No visible band appeared on the sucrose

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TABLE 1. Categorical breakdown of the 230 test sera

Classification	No. tested	Mean age (yr)	Sources or references
Measles early (6 mo) postinfection (MEPI) sera	44	21	Tahitians (4); Gorotire Indians, Brazil
Measles early (2 to 3 yr) postvaccination (MEPV) sera	50	23	Icelanders (7); Xikrin Indians, Brazil (3)
Measles Late (20+ yr) postinfection (MLPI) sera	50	31	Individuals bled at Yale-New Haven Hospital
MS sera	50	48	Multiple Sclerosis Human Neurospecimen Bank, Los Angeles, Calif.
SSPE sera	11	21	University of South Alabama Medical Center; National Institutes of Health
Acute-phase AM (AAM) sera	11	15	Provincial Laboratory of Public Health, Calgary, Alberta, Canada (13)
Convalescent-phase AM (CAM) sera collected 2 to 3 weeks after bleeding of early sera	14	15	Provincial Laboratory of Public Health (13)

cushion following high-speed centrifugation, but the interface material was collected and used as the negative control.

Western blot. The MV preparation and the negative control were run separately on a series of Laemmli sodium dodecyl sulfate-polyacrylamide gels (14 cm by 14 cm by 1.5 mm) (18) (stacking gel, 4%; separating gel, 10%) by using toothless combs. The samples were diluted 1:4 in standard sample buffer and boiled for 3 min before 4.4 ml was applied to each gel. Gels were electrophoresed at a constant current of 20 mA per gel until the dye front reached the separating gel, at which time the current was increased to 30 mA until the dye reached the bottom. At the end of electrophoresis, the stacking gel was discarded. The separating gel was equilibrated in transfer buffer (0.025 M Tris, 0.192 M glycine [pH 8.3]) and placed in a Bio-Rad electrophoretic transfer apparatus. A sheet of nitrocellulose paper was laid over the separating gel, and transfer was conducted for 3 h at 70 V. Following transfer, the nitrocellulose paper was dried, cut vertically into strips 4 mm wide, and stored in sealed containers. The shelf life of these strips was found to be approximately 2 months without a recognizable loss of antigenicity.

Reaction with sera. Sera diluted 1:20 in 1% gelatin-Tris-buffered saline (TBS) were prepared in test tubes (10 by 75 mm). An MV antigen strip which had been incubating for 1 h in a 3% gelatin-TBS blocking solution was placed in each tube. (Initially, two strips, one carrying MV antigens and the other carrying Vero cells, were added to each tube. How-

ever, because no reaction was observed on any of the Vero cell strips, this control was subsequently omitted.) The test tubes were sealed, and the strips were incubated for 18 h on a rotator at 5 rpm. The strips were then washed three times, once briefly with water and twice for 10 min each time with 0.5% Tween 20 in TBS, and exposed to 1:2,000 peroxidase-conjugated sheep anti-human immunoglobulin G (heavy and light chains) in 1% gelatin TBS. After 1 h of incubation at room temperature on a shaker platform, the strips were washed again as described above and exposed to the Bio-Rad enzyme immunoassay color development solution containing 4-chloro-1-naphthol.

To quantitate the bands on the nitrocellulose strips, a Bio-Rad model 1650 reflectance densitometer was used. For each batch of 32 MV strips cut from a single sheet of nitrocellulose paper, the densitometer sensitivity was standardized to read 50 units in peak height for the N protein antibody band of a positive control serum sample. The antibody levels themselves were determined by integrating the area under each peak according to Simpson's Rule (31) with the aid of a microcomputer program.

Serosurvey. The serosurvey was conducted with strips which were 12 to 16 days old. A total of 230 serum samples from the seven comparison groups were tested. Table 1 summarizes the categorical breakdown of the test sera (note the definitions of abbreviations for serum samples in Table 1). Each set of 32 MV strips obtained from one nitrocellulose sheet was incubated with 27 test sera, 2 measles-positive control sera, 2 measles-negative control sera, and 1 sample consisting only of buffer solution (1% gelatin-TBS). In addition, two strips with only Vero cells were incubated with the two measles-positive control sera.

Datum analysis. The Student *t* test was used to determine the significance of differences in relative amounts of antibodies to individual proteins between the different comparison groups. Although the number of sera available in some categories was small, all estimates of probability take these numbers into account.

RESULTS

Characterization of the MV Western blot. To test the assay for reproducibility, we made 10 replicate runs on one of the positive control serum samples (Table 2). These yielded only small variations in peak areas corresponding to antibodies against each of the four MV proteins. Table 2 also shows results of the test for specificity conducted on five negative control serum samples. In addition, the proportionality of peak areas to serum concentrations for each of the four MV proteins was verified in a series of tests on a positive control serum sample (Fig. 1).

TABLE 2. Actual antibody levels in positive control replicates and in negative controls

Test material	MV antibody specificity	Mean area beneath peak (mm ² ± SD)
Positive control replicates (n = 10)	Anti-H	6.0 ± 0.8
	Anti-P	44.6 ± 3.4
	Anti-N	213.8 ± 7.0
	Anti-M	33.0 ± 3.4
Negative controls (n = 6)	Anti-H	0.0
	Anti-P	0.1
	Anti-N	0.1
	Anti-M	0.0

FIG. 1. Linearity of peak areas to serum dilutions for antibody against each of the four MV proteins in the Western blot. Symbols: ●, anti-P; ×, anti-N; +, anti-M; ▲, anti-H.

Transformation of the data for statistical analyses. To emphasize qualitative rather than quantitative differences in immune responses, we decided to focus the comparisons more on the relative antibody levels than on the actual peak areas. The relative level is the proportion of the total area for all four MV polypeptides attributable to antibody against one protein. Within each of the seven serum groups, the relative values for anti-P and anti-N satisfied the statistical preconditions of normality of distribution and equality of variance (9). However, for anti-H and anti-M, transformation of the relative values to their natural logs was needed to satisfy these requirements.

Comparisons among the three control group sera. The actual levels of antibodies to each of the four MV proteins were lower in vaccinated than in naturally infected individuals, but there was no significant difference in the relative amounts of the four antibodies (Tables 3 and 4). Comparing these two groups with the MLPI group, we found that the anti-H level in MLPI sera was low in both actual and relative terms and that the anti-P level was high, although the latter was significantly different only in comparison with MEPI sera.

Comparisons among MLPI, MS, and SSPE sera. MS and SSPE sera were compared with MLPI sera (Tables 3 and 4) because patients with these two disorders usually have a history of natural measles several years prior to the onset of symptoms. The MLPI-MS comparison showed a significant difference only in the actual and relative amounts of anti-M, which was low in the MS group. In comparison with MLPI

TABLE 3. Mean actual and relative antibody levels in the seven serum groups

Classification	Actual (relative) levels of:			
	Anti-H	Anti-P	Anti-N	Anti-M
MEPI	23.5 (0.15)	27.5 (0.16)	93.2 (0.57)	21.2 (0.11)
MEPV	15.2 (0.15)	20.1 (0.17)	68.8 (0.57)	11.0 (0.11)
MLPI	7.9 (0.05)	43.2 (0.21)	121.8 (0.62)	23.9 (0.12)
MS	8.6 (0.06)	36.7 (0.19)	128.3 (0.68)	14.3 (0.07)
SSPE	28.8 (0.05)	210.0 (0.39)	245.8 (0.46)	48.2 (0.09)
AAM	8.7 (0.07)	53.8 (0.25)	133.6 (0.61)	12.1 (0.08)
CAM	24.9 (0.06)	226.1 (0.37)	215.3 (0.44)	88.5 (0.13)

sera, SSPE sera showed elevated levels of antibodies against all four MV proteins. However, in relative terms, the increase was evident only in anti-P, and there was a significant relative decrease in anti-N. Similar results were obtained when SSPE sera were compared with MS sera.

Comparisons among AAM, CAM, and MEPI sera. Since AAM and CAM sera were all collected within a few weeks of infection, the most appropriate comparison was made with MEPI sera (Tables 3 and 4). AAM sera showed low levels of anti-H and anti-M and slightly elevated levels of the other two antibodies. In relative terms, however, only the low level of anti-H and the high level of anti-P were significant. All CAM titers except the anti-H titer were high in actual terms. A comparison of the relative amounts of the four antibodies in the CAM group and the MEPI group revealed a pattern similar to that of the AAM-MEPI comparison, except that the proportion of anti-N became significantly lower. When AAM and CAM sera were compared with each other, AAM sera showed significantly lower proportions of anti-P and anti-M and a higher proportion of anti-N. In both relative and actual terms, the CAM results were strikingly similar to the SSPE results.

DISCUSSION

The MV Western blot is an assay capable of testing a large number of sera for antibodies against the major MV structural proteins. As the results of the positive control repli-

TABLE 4. Comparisons of relative antibody levels among serum groups

Serum group comparison	P value ^a for:			
	Anti-H	Anti-P	Anti-N	Anti-M
MEPI vs MEPV	NS	NS	NS	NS
MEPI vs MLPI	<0.01	<0.05	NS	NS
MEPV vs MLPI	<0.01	NS	NS	NS
MLPI vs MS	NS	NS	NS	<0.05
MLPI vs SSPE	NS	<0.01	<0.01	NS
MS vs SSPE	NS	<0.01	<0.01	NS
MEPI vs AAM	<0.01	<0.01	NS	NS
MEPI vs CAM	<0.01	<0.01	<0.01	NS
AAM vs CAM	NS	<0.05	<0.01	<0.05

^a P values were determined by the two-tailed Student *t* test. NS, Statistically not significant.

cates and negative controls show, the MV Western blot exhibits good reproducibility and specificity (Table 2). Measurements are made on a continuous arithmetic scale and are quantitatively more precise than data yielded by tests which depend on serial dilutions. The linearity of the plots of areas beneath absorbance peaks versus serum dilutions (Fig. 1) indicates that the test as performed is not subject to the prozone phenomenon or to the saturation of binding sites at high antibody concentrations. The slopes for the individual proteins differentiate amounts of antibody bound by each protein from the same serum dilutions. This slope is determined by the number of native epitopes remaining on each protein molecule and the relative effectiveness of these epitopes in eliciting antibody. It was initially a surprise to us to find that the H protein produced the lowest slope, because the most sensitive tests for antibody, neutralization, and hemagglutination inhibition are dependent on reactions with this protein. These other tests, however, involve reactions with very few epitopes (12), and inasmuch as these key epitopes represent biologically active sites, they may be more than usually subject to denaturation. Relatively weak H protein bands were also observed by Rozenblatt et al. (29) in the reaction between sodium dodecyl sulfate-denatured measles-infected cell products and anti-measles guinea pig serum, although the Haire technique yielded similar levels for H and N (14).

This limitation of the MV Western blot test, the fact that labile epitopes may be lost during immobilization of MV proteins on nitrocellulose paper, is common in at least some degree to any procedure that requires separation of the virus into its component proteins. Comparisons between the anti-H Western blot titer and the hemagglutination inhibition titer in normal individuals (data not shown) yielded only a modest Pearson product-moment correlation ($r = 0.55$), suggesting that changes had occurred. This value is, however, higher than the correlation between the same antibodies determined by hemagglutination inhibition and immunoprecipitation. Wechsler and Meissner reported $r = 0.26$ for normal sera and $r = -0.07$ for MS sera (34) by the latter methods. Comparisons of amounts of antibodies combining with the several virus proteins by immunoprecipitation would be influenced by differences in the number of methionine residues, as well as by epitope stability (26).

To assemble appropriate sets of sera with each of the diseases being considered, we found it necessary to seek them from diverse sources. Because the ages at which these diseases strike are characteristically different, the mean age differed from one group to another. We do not believe that these age and ethnic differences played important roles in determining the differences in immune responses in different syndromes. Clinical (7) and immunological (4) reactions to MV infection and to vaccines (13) differed only at extreme age ranges in situations in which all ages were involved. Antibody persistence did not differ by age at immunization (5). Reactions are commonly more severe in crowded, malnourished populations, where a very young age group may be severely affected (1), but in well-nourished groups ethnicity has not been found to play a significant role in disease manifestations (23), vaccine reactions (8), or antibody titer (F. L. Black, L. L. Berman, J. M. Borgono, R. A. Capper, A. A. Carvalho, C. Collins, O. Glover, Z. Hijazi, D. L. Jacobson, Y.-L. Lee, M. Libel, A. C. Linhares, C. A. Mendizabal Morris, E. Simoes, E. Siqueira-Campos, J. Stevenson, and N. Vecchi, *Am. J. Epidemiol.*, in press). In terms of the hemagglutination inhibition, neutralization, and complement fixation tests, even large time differences, if

more than 1 year after infection, have only a minor effect on titer (4-6).

Comparisons among the three control groups reveal remarkable similarities in the responses to vaccine and to disease but some change over time. MEPI and MEPV sera are indistinguishable with respect to relative antibody levels against the four MV proteins. Thus, the live attenuated vaccine is able to elicit an antibody balance against the MV proteins which closely mimics that of the natural disease. In both MEPI and MEPV sera, there is rapid early production of antibody against a viral component (H) known to be important in controlling the spread of virus, whereas the production of antibody against a protein (P) which has no demonstrated role in controlling virus growth increases after the acute phase of the disease. An increase in the production of antibody against this nonglycosylated protein apparently continues after infectious virus has been cleared from the circulation. The persistence of measles antibody measured by hemagglutination inhibition or complement fixation over decades without restimulation has long been an enigma (6; J.-F. Lian, Ph.D. dissertation, Yale University, New Haven, Conn., 1979). Now we find that the production of antibody to the P protein actually increases after apparent termination of the acute infection. The inference of the persistent titers has always been that antigenic stimulus must continue after the disease has ended, but specific evidence of latent infection has not been found except in SSPE. The observed increase in the production of antibody to the P protein long after infection compounds this problem but creates no new one.

The MS patients had presumably been exposed to MV some years earlier, and their relative H, P, and N titers were comparable to those of the MLPI individuals. However, antibody to the M protein was significantly reduced in these patients. In this series we did not see an absolute increase in antibodies against the other MV proteins in MS patients; the increase observed in other studies is small and statistically significant only with large numbers of subjects (2, 14). The relative reduction in M protein antibody was greater than would have been produced by the commonly reported absolute increase in the other antibodies. Whatever the underlying mechanism is, this qualitative difference in MV antibody levels between the two groups provides one more link between MV and MS.

A comparison between SSPE and MLPI sera shows differences in relative levels of anti-P and anti-N. These findings are also observed in the comparison between SSPE and MS sera, and they emphasize the distinct roles of MV in these two diseases. The SSPE antibody profile generated in this study differs from those generated in studies with immunoprecipitation. The earlier studies reported a lack of anti-M in SSPE sera (15, 35), and it was hypothesized that this deficit resulted from the inability of the infecting MV to produce the M protein. We found normal relative levels of anti-M in SSPE sera. Although inconsistent with the earlier reports, this finding is supported by the results of a recent study by Norrby et al. (24) which showed the presence of the M protein in SSPE biopsy material. Ohara et al. attribute the diverse immunoprecipitation results with anti-M to differences in the buffers (27). On the basis of a conservative hypothesis, one may prefer to suppose that the M protein epitopes were poorly retained by immunoprecipitation rather than that the H, P, and N protein epitopes were less efficiently preserved by the Western blot.

The CAM sera show actual levels of MV antibodies comparable to the very high levels observed in SSPE sera.

Both the AAM and CAM sera show lower relative levels of anti-H than do MEPI sera. Antibody to this protein is of greatest importance in virus neutralization, and it is possible that the high levels of sensitization to the N, P, and M proteins relative to the H protein played a role in the more severe disease. Unfortunately, we were not able to produce reliable data for anti-F, to which a critical role for virus spread has been attributed by *in vitro* studies (21).

We set out to determine the relative response to M protein in different measles-related diseases and found that it is depressed in MS patients but not in SSPE patients relative to long-term naturally immune persons. In the process we have encountered two new problems. (i) What does the deficit in anti-M in MS patients signify? Such a situation may arise if M protein production or anti-M response is suppressed because of unusual virus or host genetics; in either case viral latency might be accentuated. On the other hand, the deficit in anti-M may be due to its removal by enhanced production of the analogous anti-idiotypic. Excess anti-idiotypic could react with cell receptors for the M protein and cause impairment of nerve function. (ii) What role does the P protein play in viral pathogenesis and recovery? The relatively high and late development of anti-P has not been documented previously. This pattern would seem to imply that the P protein persists in the body longer or in relatively greater amounts than do other MV proteins, and the old question of continued production by latent virus is renewed. More studies are needed to solve both of these problems.

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