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NOTES

Search for Cytomegalovirus-Specific Immunoglobulin M: Comparison between a New Western Blot, Conventional Western Blot, and Nine Commercially Available Assays

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We tested 101 serum samples obtained from pregnant women for the presence of human cytomegalovirus (HCMV)-specific IgM with nine different commercially available kits and with two Western blotting (WB) tests previously developed in our laboratory. The conventional WB test contains viral structural proteins separated on a gel from purified HCMV particles and transferred to nitrocellulose. The new WB test contains viral structural proteins and three recombinant proteins which contain the significant immunogenic portions of pp150 (ppUL32), pp52 (ppUL44), and p130 (pUL57). The results obtained indicate that the new WB test combines high specificity (92.5%) with high sensitivity (95.0%), characteristics that, in combination, have not been obtained with any of the other tests.

Immunoglobulin M (IgM) to human cytomegalovirus (HCMV) can be detected by a variety of different procedures, but detection has been hampered by technical problems causing interassay variability. The most widely used procedure for IgM detection is enzyme immunoassay (EIA) (12, 16–19). Many different assays for HCMV IgM are commercially available, but poor agreement has been found among the results obtained by testing different kits (8).

Western blotting (WB) by using viral polypeptides separated from purified viral particles has repeatedly been shown to be a reliable and sensitive method for detection of HCMV-specific IgM (1, 3, 8). Recently, we studied the correlation between an active HCMV infection determined by virological parameters (isolation of the virus from urine and/or saliva from immunocompetent subjects and determination of the presence of HCMV pp65 or the viral genome in polymorphonuclear leukocytes from immunocompromised patients) and the presence of HCMV-specific IgM as detected by WB (9). The agreement observed between IgM detection by WB and the results obtained by virological detection of HCMV was significantly higher (88.7%) than the agreement of IgM detection by one of the many commercially available EIA kits with virological results (67.5%). However, in this study some false-positive WB results were observed mainly when a serum showed reactivity exclusively to pp150, very likely because two proteins overlap at that molecular weight (ppUL32 and pUL86) and one of them (pUL86) is the herpesvirus group viral capsid antigen. Furthermore, the sensitivity of the assay, although good, could still be improved. Of the HCMV nonstructural proteins, at least two have been shown to react with IgM very efficiently. These proteins are ppUL44 (pp52), an abundant nuclear phosphoprotein which is essential for HCMV replication and binds to the viral DNA polymerase, and pUL57(p130), another DNA-binding protein with an unknown function (2). These proteins are not present in the conventional WB test based on viral structural proteins (convWB).

To overcome these two problems, we recently developed a novel WB test (newWB) for anti-HCMV IgM detection which contains viral structural polypeptides and significant portions of recombinant pp150 (ppUL32) to confirm the positivity of sera reacting exclusively with pp150 and two recombinant nonstructural proteins (a significant portion of ppUL44 and the two immunodominant regions of pUL57) to capture IgM reactivity to nonstructural proteins. This new test was evaluated with latently infected blood donors, pregnant women, and transplant recipients with ongoing HCMV infections (10) and proved more specific and sensitive than the conventional EIA (convEIA) routinely used in our diagnostic laboratory. As expected, the results from this new test correlated much better with the virological test results than did the convEIA results. We wanted to know how well the results obtained with this newWB, which agree very well with virological test results, would agree with the results obtained from a consensus of nine different HCMV IgM tests. In the present work, we compared the performance of the newWB with that of the convWB and nine commercially available kits for the detection of HCMV-specific IgM.

A total of 101 serum samples obtained from pregnant women were used. Serum samples were sent to our diagnostic center from other Italian laboratories for confirmation of the presence of HCMV-specific IgM because they gave either borderline values or discordant results with two different tests. Samples were stored frozen at −20°C until tested. Sera were tested for the presence of rheumatoid factor by latex agglutination (Rheuma-Wellco test; Wellcome, Dartford, England), and only rheumatoid factor-negative sera were included in this study.

The Towne strain of HCMV was used for the WB tests. The

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virus was propagated in human embryo fibroblasts by using standard methods and purified by a sorbitol cushion followed by a sorbitol gradient as previously described in detail (4).

For the newWB, the following *Escherichia coli* CTP:CMP-3-deoxy-D-manno-octulosonate cytidylyl synthetase (CKS) recombinant proteins were used: (i) two ppUL32 regions (amino acids [aa] 595 to 614 and 1006 to 1048) fused together, which can replace the IgM-binding ability of the entire pp150 molecule (13); (ii) the carboxy-terminal part of ppUL44 (aa 202 to 434), which contains epitopes highly reactive with IgM and does not contain relevant amino acid sequences cross-reacting with the homologous protein of other members of the *Herpesviridae* family (14); and (iii) two segments of pUL57 (aa 540 to 601 and 1144 to 1233) cloned separately and previously shown to be highly reactive with serum IgM (11, 20). The recombinant proteins described above were obtained, characterized, and purified as previously described in detail (7, 15).

HCMV serologic testing was done by following methods. (i) The convEIA was done with the following commercial kits: CMV IgM ELISA (Wampole Laboratories, Cranbury, N.J.), CMV-IgM ELA test (Technogenetics, Hamburg, Germany), ELISA CMV IgM (Sclavo, Siena, Italy), BEIA CMV-IgM (Bouty Diagnostici, Milan, Italy), CMV IgM ELISA (Eurogenetics N.V., Tessenderlo, Belgium), ETI-CYTOK-M (Sorin Biomedica, Vercelli, Italy), Enzygnost Anti-CMV IgM (Behringwerke AG, Marburg, Germany), CMV STAT (M.A. Bioproducts, Walkersville, Md.), and Cytomegaly-virus ELISA IgM (Human Gesellschaft für Biochemica und Diagnostica, Taunsstein, Germany). All tests were performed and the results were interpreted as suggested by the manufacturers. (ii) For the convWB, protein extracts from purified viral particles were run on a 9% polyacrylamide gel; electrophoretically separated polypeptides were then transferred to nitrocellulose paper. Infection of cells, virus purification, protein extraction, blotting, and immune reaction with sera were done as previously described in detail (6). (iii) The procedure for construction of the newWB has recently been described in detail (10). Briefly, lysates of purified virions were run on preparative gels. Separated polypeptides were transferred to nitrocellulose sheets by standard WB as already described (5). The nitrocellulose sheets used for transfer of viral proteins exceeded the length of the polyacrylamide gels to leave enough space at the bottom for the recombinant proteins. After the electrophoretic transfer of viral proteins from the gel, each blot was mounted on a Miniblotter apparatus (Biometra, Göttingen, Germany) so that the channels of the Miniblotter were oriented in the same direction as the bands of the transferred proteins. Suspensions of the recombinant proteins (pp150, pp52, and pp130) were deposited in the Miniblotter wells. In addition to the recombinant proteins, two further control proteins were deposited onto the nitrocellulose. The CKS protein was added as a negative control to monitor for the presence of serum IgM to the bacterial portion of the fusion protein. Human μ chain (IgM) was added as a positive control to monitor the reaction of the conjugate to human IgM. Miniblotters were then gently agitated on a rocking platform overnight at room temperature. The filters were washed briefly in Tris-buffered saline (TBS) and then saturated by incubation with a blocking solution (3% fish gelatin, 1% bovine serum albumin, 5% powdered skimmed milk, 0.05% Tween 20 in TBS) at room temperature for 1 h. The filters were then cut into 3-mm-wide strips carrying both authentic viral proteins (at the top) and recombinant polypeptides (at the bottom); each resulting strip consisted of a combination of the convWB and a recombinant blot.

Serum samples were diluted 1:50 in TBS with 4% fetal calf serum and 0.1% Tween 20 and incubated at room temperature for 3 h. After three washes with phosphate-buffered saline–Tween 20, peroxidase-conjugated anti-μ chain antibodies (KPL, Gaithersburg, Md.) diluted 1:5,000 in TBS with 10% fetal calf serum was added and the mixture was incubated at room temperature for 1.5 h. Washing, development, and interpretation of the results were performed as previously described (10).

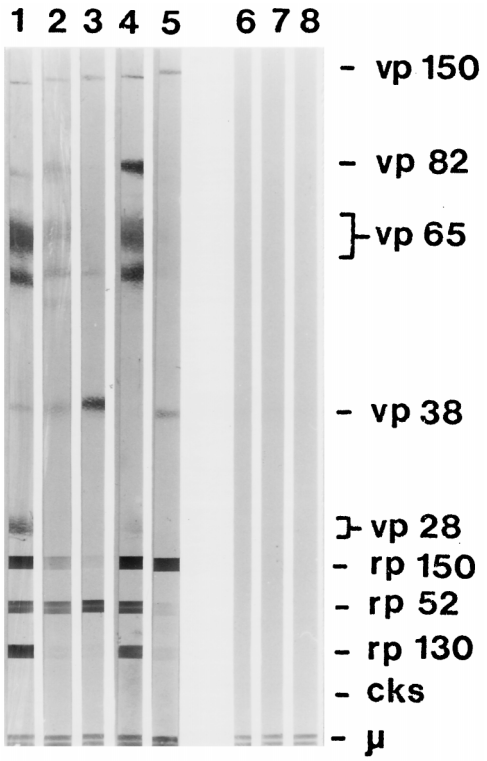

FIG. 1. Representative examples of serum reactivity with the newWB. Values on the right are molecular weights (10^3) of viral (vp) or recombinant (rp) proteins. CKS is the negative control; μ is the IgM heavy chain and represents the positive control. Lanes 1 to 5 contained IgM-positive sera and lanes 6 to 8 contained IgM-negative sera from pregnant women.

Some representative examples of the reactivity of human sera from pregnant women with the newWB are shown in Fig. 1. The samples in lanes 1 to 5 were positive for HCMV-specific IgM, as at least one reactive band was present in the viral section and the recombinant section of the blot. The samples in lanes 6 to 8 were negative for HCMV-specific IgM, as no bands were present in either section of the blot. A positive reaction with human μ chain and no reaction with the CKS carrier protein in all lanes confirmed the validity of the assay. Of 101 serum samples tested by nine different EIA kits, only 37 (36.6%) gave identical (positive or negative) results with all of the kits. We classified these serum samples, sent to our laboratory for confirmation, as positive or negative for HCMV-specific IgM based on the concordance of results from at least six or more kits. Serum samples with a concordance of results from fewer than six kits were classified as indeterminate. Based on concordance of results from at least six of the nine kits, 39 (38.6%) serum samples were judged positive and 40 (39.6%) were defined as negative. Twenty-two serum samples (21.8%) giving less than six concordances were defined as IgM indeterminate.

Table 1 summarizes the sensitivity, specificity, and overall agreement, as well as false-positive and -negative result rates,

TABLE 1. Comparison of the results obtained with nine commercial EIA kits for detection of HCMV IgM

Assay[a]	Total no. of results		No. of true results		No. of false results		SNC[b]	SPE[c]	Overall agreement (%)[d]
	Positive	Negative	Positive	Negative	Positive	Negative			
1	58	43	39	38	19	5	88.6	66.6	76.2
2	59	42	33	35	26	7	82.5	57.4	67.3
3	21	80	21	40	0	40	34.4	100	60.3
4	21	80	18	39	3	41	30.5	92.9	56.4
5	54	47	36	37	18	10	78.3	67.3	72.3
6	52	49	38	39	14	10	79.2	73.6	76.2
7	30	71	26	40	4	31	45.6	90.9	65.3
8	65	36	39	31	26	5	88.6	54.4	69.3
9	48	53	37	40	11	13	74	78.4	76.2

[a] 1, CMV IgM ELISA (Wampole Laboratories, Cranbury, N.J.); 2, CMV-IgM ELA test (Technogenetics, Hamburg, Germany); 3, ELISA CMV IgM (Sclavo, Siena, Italy); 4, BEIA CMV-IgM (Bouty Diagnostici, Milan, Italy); 5, CMV IgM ELISA (Eurogenetics N.V., Tessenderlo, Belgium); 6, ETI-CYTOK-M (Sorin Biomedica, Vercelli, Italy); 7, Enzygnost Anti-CMV IgM (Behringwerke AG, Marburg, Germany); 8, CMV STAT (M.A. Bioproducts, Walkersville, Md.); 9, Cytomegaly-virus ELISA IgM (Human Gesellschaft für Biochemica und Diagnostica, Taunsstein, Germany).
[b] SNC, sensitivity. SNC = (true positives)/(true positives + false negatives) × 100.
[c] SPE, specificity. SPE = (true negatives)/(true negatives + false positives) × 100.
[d] Overall agreement = (true positives + true negatives)/(total number tested) × 100.

for each kit determined on the basis of the agreement among at least six kits. Sensitivity ranged from 30.5 to 88.6%, specificity ranged from 54.4 to 100%, and overall agreement ranged from 56.4 to 76.2%. These results are in agreement with previous data (8) demonstrating the detection of HCMV-specific IgM in samples from pregnant women.

All of these sera were then tested by convWB, as well as with the newWB; the results obtained are shown in Table 2. Of 39 serum samples that were judged IgM positive by at least six different assays, 37 were positive by the newWB while only 25 were positive by the convWB. Furthermore, of 40 serum samples that were defined as negative because at least six assays found them negative, the convWB detected four positives while only three were found positive by the newWB. Of 20 serum samples that were considered EIA indeterminate, the convWB found 12 positive, 7 negative, and 3 indeterminate, while the newWB found 13 positive and 9 negative.

From these results, the sensitivity and specificity of the two WB tests were calculated relative to the consensus. The sensitivity and specificity of the convWB were 64.1 and 80.0%, respectively, whereas the sensitivity and specificity of the newWB were 95.0 and 92.5%. Therefore, the newWB combines high sensitivity with high specificity, characteristics that are mutually exclusive with the other commercially available tests. Furthermore, the results obtained with the newWB agree with the results of the consensus of nine commercially available HCMV IgM assays. This result, combined with the good correlation of the newWB to virologic test results (10), establishes the newWB as a benchmark for performance of HCMV IgM serological assays.

Kits available on the market use antigenic materials composed of proteins extracted from virus-infected cells (composed of a mixture of 50 to 100 structural and nonstructural antigens) or purified viral particles (composed of a mixture of 30 to 55 structural proteins). If more than one of these kits is used, discordant results are obtained in 20 to 40% of the cases and a test based on the use of some carefully chosen recombinant proteins could be of some help as a confirmatory test. A

TABLE 2. Results obtained by testing 101 serum samples with the convWB and the newWB

No. of positive concordances	ELISA score	No. of samples	No. of convWB results			No. of newWB results	
			Positive	Negative	Indeterminate	Positive	Negative
9	Positive	10	9	1	0	10	0
8	Positive	4	3	0	1[a]	4	0
7	Positive	15	8	7	0	14	1
6	Positive	10	5	5	0	9	1
Totals:		39	25	13	1	37	2
5	Indeterminate	12	6	4	2[b]	8	4
4	Indeterminate	10	6	3	1[b]	5	5
Totals:		22	12	7	3	13	9
3	Negative	2	2	0	0	2	0
2	Negative	4	1	3	0	1	3
1	Negative	7	1	4	2[b]	0	7
0	Negative	27	0	25	2[b]	0	27
Totals:		40	4	32	4	3	37

[a] Reactivity to vp 82 alone.
[b] Reactivity to vp 150 alone.

second generation of kits for the detection of HCMV-specific IgM that will become commercially available in the near future contains antigenic materials composed of selected recombinant proteins. In this case, different manufacturers will use different recombinant proteins and different regions of the same protein. Therefore, it is conceivable that discordance between recombinant tests and between viral and recombinant tests may occur.

Although detection of the virus in pathological material should always be considered the "gold standard" for identification of acute HCMV infection, there are several reasons for inability to detect the virus, for example, intermittent virus shedding, virus elimination by a different route, improper storage of pathological material, etc. Therefore, a serological gold standard will be important. A test such as the newWB that we have devised, containing both whole viral structural proteins and the two major nonstructural proteins obtained as recombinant proteins, is promising in this respect. Results obtained with this test have been shown to agree well with virologic test results (10) and with results obtained from the consensus of nine commercially available tests. The newWB test can be used as a benchmark for the performance of HCMV IgM serological assays and hence as a useful tool for the development of more reliable immunoassays for the detection of HCMV-specific IgM. A large-scale screening of patients who are also monitored virologically is necessary to determine whether the newWB can be considered a reference standard for HCMV IgM serologic tests.

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