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A Novel Western Blot Test Containing Both Viral and Recombinant Proteins for Anticytomegalovirus Immunoglobulin M Detection

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We devised a novel Western blot (WB) test for anti-human cytomegalovirus (HCMV) immunoglobulin M (IgM) detection which contains viral structural polypeptides, significant portions of recombinant p150 (ppUL32), and a significant portion of the most immunogenic nonstructural protein p52 (ppUL44). This new test was evaluated in latently infected blood donors, pregnant women, and transplant recipients with ongoing HCMV infection and shown to be more sensitive and specific than traditional WB and conventional enzyme immunoassay for the detection of HCMV-specific IgM.

Human cytomegalovirus (HCMV)-specific immunoglobulin M (IgM) is a sensitive and specific indicator of primary HCMV infection in immunocompetent subjects and is often produced during active viral reactivation in immunocompromised patients (1, 4, 17, 19, 24). Serum IgM to HCMV can be detected by a variety of different procedures, but its detection has been hampered by several technical problems causing interassay variability. The most widely used procedure for IgM detection is enzyme immunoassay (EIA) (18, 24–27). Many different EIAs for HCMV IgM are available commercially, and poor agreement has been found among the results obtained with different EIA kits (13). Western blots (WB) employing viral polypeptides separated from purified viral particles has repeatedly been shown to be a reliable and sensitive method to detect HCMV-specific IgM (1, 6, 8, 13). Recently, we studied the correlation between an active HCMV infection determined by virological parameters (isolation of the virus from urine and/or saliva samples from immunocompetent subjects and determination of the presence of HCMV pp65 or the viral genome in polymorphonuclear leukocytes (PMNL) in immunocompromised patients) and the presence of HCMV-specific IgM as detected by WB (15). The agreement observed between IgM detected by WB and the results obtained by virological detection of HCMV was significantly higher (88.7%) than the agreement of IgM detected by one of the many commercially available EIA kits and virological results (67.5%).

In this study some WB false-positive results were observed when a serum sample exhibited reactivity exclusively to pp150. This could be due to the fact that two proteins overlap at that molecular weight (ppUL32 and pUL86) and that one of these proteins (pUL86) is the herpesvirus group common antigen. To avoid such a false-positive result, a serum sample reacting exclusively with p150 should be confirmed with a recombinant antigen containing significant epitopes of ppUL32.

The sensitivity of the WB assay could be improved. In fact, of 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 of unknown function (3). These proteins are not present in the conventional WB (convWB) which contains only viral structural proteins.

Recombinant antigens containing significant portions of ppUL32 (rp150), ppUL44 (rp52), and pUL57 have been obtained by us and by other researchers and evaluated serologically (12, 16, 27, 28). The aim of the present work was the development, optimization, and preliminary clinical evaluation of an ideal serological test for detection of HCMV-specific IgM. This novel WB (newWB) is based on the convWB to which purified recombinant proteins have been added. In particular, significant portions of recombinant p150 (ppUL32) have been added in order to confirm a positive result exclusively for viral p150 and a significant portion of the most immunogenic nonstructural protein p52 (ppUL44) has been added, as nonstructural proteins are not present in the purified preparations of viral particles. The further addition of the ppUL57 protein was evaluated but not deemed essential because the addition of this protein did not increase the sensitivity of the test.

MATERIALS AND METHODS

Virus and cells. The Towne strain of HCMV was propagated in human embryo fibroblasts by standard methods. The virus was purified by use of a sorbitol cushion and then a sorbitol gradient as previously described in detail (9).

Recombinant proteins. The following *Escherichia coli* CMP–2-keto-3-deoxyoctulosonic acid (CMP-KDO) synthetase (CKS) recombinant proteins were used: (i) two ppUL32 regions (amino acids [aa] 595 to 614 plus 1006 to 1048) fused together which can replace the entire p150 molecule in its IgM-binding ability (21); (ii) the carboxy-terminal part of ppUL44 (aa 202 to 434), which contains highly reactive epitopes for IgM and does not contain relevant amino acid sequences cross-reacting with the homologous protein of other members of the *Herpesviridae* family (22); and (iii) two segments of ppUL57 (aa 540 to 601 and 1144 to 1233) previously shown to be very reactive with serum IgM (16, 28). The recombinant proteins described above were obtained and characterized as previously described (12).

Insoluble CKS-HCMV fusion proteins were initially purified after lysis by a combination of detergent washes followed by solubilization in 8 M urea (23). After solubilization in 8 M urea, the fusion proteins were purified by Q-Sepharose chromatography (Pharmacia Biotech, Piscataway, N.J.).

Serum samples. A total of 430 sera were used in this work. Of the 430 sera, 200 sera were from 200 randomly selected and HCMV-seropositive blood donors (obtained through the courtesy of the Blood Transfusion Center of the St. Orsola General Hospital, Bologna, Italy), 60 IgM-positive sera were from immunocom-

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petent subjects (mainly pregnant women and healthy adults), 59 sera were from 59 pregnant women with or without HCMV infection, and 111 samples were from immunocompromised patients (3 renal transplant recipients and 21 heart transplant recipients).

Diagnosis of active HCMV infection. (i) Immunocompetent subjects. For immunocompetent subjects, the presence of an active HCMV infection was documented on the basis of virus isolation from urine and/or saliva by the shell-vial procedure (5). The inoculated cells were fixed 24 to 48 h after inoculation and stained in an indirect immunofluorescence (IIF) assay using a monoclonal antibody reacting with HCMV immediate-early 1 or 2 gene product (E13 from Argene, Varilhes, France).

(ii) Immunocompromised patients. For immunocompromised patients, antigenemia and/or PCR on PMNL was performed. The presence of HCMV pp65 (ppUL83) in PMNL (antigenemia) of immunocompromised patients was determined as originally described by van der Biji et al. (25) and modified by Revello et al. (20) using an HCMV pp65-specific pool of two monoclonal antibodies (1C3 and AYM-1 from Argene) in IIF tests. The presence of the HCMV genome in PMNL of immunocompromised patients was detected by PCR on 5×10^5 PMNL as previously described (14).

(iii) Pregnant women. HCMV infection in pregnant women was determined by one or more of the following parameters: virus isolation from urine, saliva, and blood samples and seroconversion for anti-HCMV antibodies.

(iv) Newborns. A congenital HCMV infection in a newborn was determined by HCMV isolation from urine during the first week of life.

HCMV serology. (i) Conventional EIA (convEIA). The evaluation of anti-HCMV IgG was carried out with a commercial kit (Enzygnost Anti-HCMV/IgG EIA alpha method; Behring AG, Marburg, Germany). Plates were read on a microEIA automatic reader (Behring AG). The evaluation of anti-HCMV IgM was performed with the Enzygnost Anti-HCMV/IgM kit (Behring AG). Both kits were used, and the results were interpreted as suggested by the manufacturers.

(ii) ConvWB. Protein extracts from purified viral particles (Towne strain) were run on a 9% polyacrylamide gel, and 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 (11).

(iii) NewWB. Lysates of purified virions were run on preparative gels. Separated polypeptides were transferred to nitrocellulose sheets by standard WB as previously described (10). The nitrocellulose sheets used for transfer of viral proteins were about one-fourth longer than the polyacrylamide gels in order to leave enough space at their bottom for the recombinant proteins. After the electrophoretic transfer of viral proteins from the gel, each blot was mounted on a Miniblotter apparatus (Biometra, Goettingen, Germany) so that the channels of the miniblotter were oriented in the same direction as the bands of the transferred proteins. Suspensions of the three recombinant proteins (p150, p52, and pUL57) were deposited in the miniblotter wells.

In addition to the recombinant proteins, two additional control proteins were deposited onto the nitrocellulose sheets. 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 kept on a rocking platform and gently agitated 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 skim milk, and 0.05% Tween 20 in TBS) at room temperature for 1 h. The filters were then cut in 3-mm-wide strips, carrying both viral authentic proteins (at the top) and recombinant polypeptides (at the bottom), each resulting strip consisting of a combination of the convWB and a recombinant dot 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 containing Tween 20, peroxidase-conjugated anti-μ chain antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:5,000 in TBS with 10% fetal calf serum were added and incubated at room temperature for 1.5 h.

Some representative examples of results with the newWB are shown in Fig. 1 and 2.

RESULTS

Reactivity with sera from blood donors. Several different combinations of IgM reactivity were observed in sera from randomly selected blood donors. The reactivity observed against viral proteins was higher than that observed with recombinant proteins. The highest reactivity was observed against viral p150 (vp150) alone (5.5%), followed by vp82 (1.5%) and vp38 (1.0%). A few sera (1.5%) reacted exclusively with recombinant p150 (rp150), and a much lower number of sera reacted with viral and recombinant proteins (1% with vp38 and rp150 and 1% with vp150, vp82, and rp150).

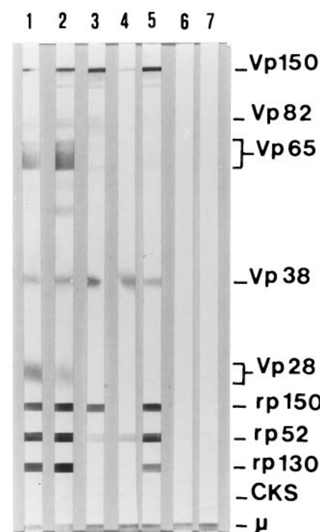

FIG. 1. Representative examples of serum reactivity with the new WB. Lanes: 1 to 3, IgG- and IgM-positive sera from pregnant women (EIA); 4 and 5, IgG- and IgM-positive sera from renal transplant recipients (EIA); 6 and 7, IgG-positive, IgM-negative sera from pregnant women (EIA). The proteins are shown to the right of the gel and were named as follows: the molecular weight (in thousands) follows the viral (Vp) or recombinant (rp) protein designation. CKS is the *E. coli* CMP-KDO synthetase and is the negative control; μ is the IgM heavy chain and is the positive control.

Reactivity with IgM-positive sera. Sixty human sera that were judged IgM positive by both convEIA and convWB were assayed by newWB. More than 50% of the sera were shown to react with two (p150 and p38) or three (p150, p65, and p38 or p150, p82, and p65) viral proteins and both recombinant proteins. It was noteworthy that not a single serum reacted with

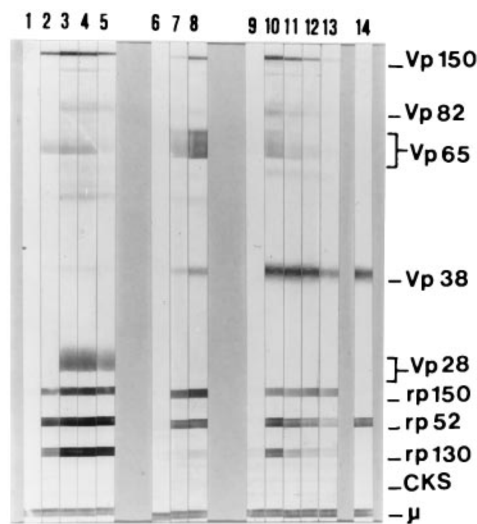

FIG. 2. Follow-up of pregnant women with primary infection (virus was transmitted to the fetus) with the new WB. Sera in lanes 1, 6, and 9 were IgG- and IgM-negative first serum samples. Sera in lanes 2, 7, and 10 were the corresponding IgG-negative and IgM-positive second serum samples. The other sera, IgG and IgM positive, were taken at 2-week intervals. Lane 14 contains an IgG-positive and IgM-negative serum sample taken from a newborn with viruria. The proteins are shown to the right of the gel and were named as follows: the molecular weight (in thousands) follows the viral (Vp) or recombinant (rp) protein designation. CKS is the *E. coli* CMP-KDO synthetase and is the negative control; μ is the IgM heavy chain and is the positive control.

TABLE 1. Comparison of convEIA, convWB, and newWB results for pregnant women[a]

Subjects	No. of subjects	No. of IgM-positive samples by:		
		ConvEIA	ConvWB	NewWB
HCMV-infected pregnant women who did not transmit the infection	30	16	18	17
HCMV-infected pregnant women who transmitted the infection	11	6	11	11
HCMV-uninfected pregnant women	18	0	1	0

[a] Samples were obtained from the women at 22 to 24 weeks of gestation.

TABLE 2. Presence of HCMV IgM detected by three methods in 18 transplant patients with symptomatic HCMV infections at the onset of symptoms

Samples[a]	No. of positive samples by:				
	ConvEIA	ConvWB	NewWB	AG[b]	PCR[b]
First (n = 18)	12	13	14	15	18
Second (n = 14)	11	12	12	11	14

[a] First, the first serum sample obtained when the symptoms appeared; second, serum sample obtained 3 to 7 days later, during treatment.
[b] Antigenemia (AG) and PCR were carried out on PMNL.

viral or recombinant proteins exclusively. Representative examples of serum reactivity with the newWB are shown in Fig. 1 and 2.

Algorithm for the interpretation of the newWB. On the basis of the results obtained with sera from latently infected blood donors and those which showed the presence of HCMV-specific IgM by two different detection methods, we determined that the newWB assay result had to be considered positive when both sections of the strip (the section containing viral proteins and the section containing recombinant proteins) gave a positive result with serum IgM. In contrast the assay was considered negative if one of the two sections of the strip was completely negative, irrespective of how many bands were reactive in the remaining section of the strip. Furthermore, both a positive reaction with the human μ chain and no reaction with the carrier protein were necessary to confirm the validity of the assay.

By using this algorithm, 2 of 200 serum samples from blood donors (1.0%) were positive for HCMV-specific IgM and all 60 IgM-positive sera were positive for HCMV-specific IgM by the newWB test.

NewWB test on sera from pregnant women. Table 1 compares the results obtained by newWB on 59 sera from a group of pregnant women with the results obtained by convEIA and convWB. Of 18 HCMV-uninfected pregnant women, none was found IgM positive by either convEIA or newWB, while a false-positive result was found by convWB. In contrast, of 30 HCMV-infected pregnant women who did not transmit the infection to their offspring, convWB detected IgM in 18 women, newWB detected IgM in 17 women, and convEIA detected IgM in 16 women. The serum sample with a negative result by newWB and a positive result by convWB was from a woman excreting the virus in urine and therefore should be considered a false-negative result for newWB. The same is true for the two sera that were found IgM negative by convEIA. Of 11 HCMV-infected pregnant women who transmitted the infection, all 11 were found IgM positive by both convWB and newWB and only 6 were IgM positive by convEIA.

NewWB test on sera from transplant recipients. NewWB, convWB, and convEIA were also compared in their ability to detect HCMV-specific IgM in a group of 24 transplant patients with HCMV infection. The patients were also monitored by the pp65 antigenemia test and by PCR for the detection of the HCMV genome in PMNL. Of the 24 patients, PCR detected a HCMV infection in 23 patients and antigenemia in 24 patients, convWB and newWB detected infection in 22 patients, and convEIA detected infection in 18 patients (data not shown). A patient who was PCR negative and antigenemia positive (only 1 pp65-positive PMNL/2 $\times$ 10^5 cells) was retested by PCR, and the negative result was confirmed, indicating the possible presence of DNA polymerase inhibitor.

No aspecific reactions could be detected by the newWB on blood samples obtained from 20 patients before infection (these samples were all PCR and antigenemia negative and were obtained 0 to 12 days after transplantation), while convEIA and convWB gave one false-positive reaction (different subjects). At the very beginning of infection, when only PCR could detect the HCMV genome in PMNL (seven patients 14 to 40 days after transplantation), newWB and conv-WB detected IgM in one patient, and another patient who turned out to be the same patient found IgM false-positive result in the previous group was judged IgM positive by convEIA. When a PCR positive result was accompanied by a positive result by the antigenemia assay (23 cases, from 10 to 78 days after transplantation), newWB detected 11 cases, convWB detected 12 cases, and convEIA detected 10 cases. Later during infection when a PCR positive result was still accompanied by a positive result by the antigenemia assay (21 patients), convEIA and convWB detected IgM in 10 and 14 patients, respectively, while 15 patients were positive by newWB. For 12 patients, we obtained a third sample at 36 to 82 days after transplantation that was still positive by antigenemia and PCR. Of these 12 samples, 11, 10, and 8 were IgM positive by convWB, newWB, and convEIA, respectively. At the end of the acute infection, when antigenemia gave a negative result and PCR was still positive (20 patients 42 to 158 days after transplantation), convEIA, convWB, and newWB detected IgM in 14, 18, and 18 patients, respectively. Finally, at the end of infection (69 to 185 days after transplantation) when PCR and antigenemia were both negative (eight patients), convEIA detected IgM in five patients, while newWB detected IgM in six patients and convWB detected IgM in seven patients.

Of the 24 transplant recipients with an HCMV infection (9 primary and 15 secondary infections), 18 experienced a symptomatic infection (8 primary and 10 secondary infections) and were treated with gancyclovir. Table 2 shows the IgM detection by convEIA and newWB when symptoms appeared. In the first serum sample taken when symptoms appeared (18 patients), convEIA detected IgM in 12 patients, while the newWB detected IgM in 14 patients. Antigenemia gave positive results for 15 patients, and PCR gave positive results for all 18 patients. We obtained samples from 14 patients 3 to 7 days after the onset of symptoms. PCR and antigenemia gave positive results in 14 and 11 cases, respectively, while convEIA and newWB detected IgM in 11 and 12 cases, respectively.

DISCUSSION

In this article we describe a novel WB-based test for anti-HCMV IgM detection which includes structural viral proteins

directly obtained from purified viral particles and recombinant proteins obtained by molecular biology.

We developed this novel WB test to improve WB assay specificity. The convWB test with viral structural proteins separated from purified viral particles (that we have routinely used since 1985) demonstrates some aspecificity when an IgM reactivity exclusively to p150 is detected. This is very likely due to the fact that at 150 kDa the herpesvirus group antigen (coded by UL86) comigrates. We thus added at the bottom of the viral blot a recombinant protein containing significant portions of ppUL32 (p150). Furthermore, since the nonstructural protein pp52 (UL44) has repeatedly been shown to react very efficiently with IgM, we also included in this novel WB a recombinant protein containing a significant portion of p52. The further addition of pUL57 was evaluated but deemed useless. Therefore, the newWB consists of a strip divided into two: an upper portion containing viral proteins and a lower portion containing recombinant proteins. Furthermore, two control proteins were also included: a positive control containing μ chains of human gamma globulin and a negative control containing the bacterial protein CKS alone.

In testing sera from blood donors and IgM-positive sera, we observed that 99% of the sera from blood donors either did not show any reactivity to the proteins present in the newWB or showed some reactivity to proteins in only one of the two sections of the blot. On the other hand, 100% of the IgM-positive sera reacted with proteins present in both portions of the newWB. For this reason we decided to assign a positive result exclusively to a serum sample with reactivity to at least one band in the recombinant section of the blot and at least one band in the viral section of the blot. By using this algorithm, 1% of sera from blood donors appear to have HCMV-specific IgM, as detected by the newWB, which is consistent with the normal rate of infection in healthy adults (18, 19). Furthermore, 100% of the IgM-positive sera were shown to be positive by newWB, indicating a very high sensitivity of the novel test.

In the first part of the study, we observed that 5% of the sera from blood donors showed reactivity to vp150 alone, which was not confirmed by reactivity with recombinant proteins, thus supporting their false-positive status. Almost 10% of the IgM-positive sera which gave a positive reaction with viral p150 alone and for this reason had to be considered IgM undetermined on the basis of the convWB, when tested with the newWB showed reactivity with recombinant antigens, thus supporting their true-positive status. Hence, the sensitivity and specificity of the newWB are higher than those for the convWB. In a group of HCMV-infected pregnant women, the newWB detected 57% of those who did not transmit the infection to their offspring and 100% of those who transmitted the infection (they were all primary maternal infections). No aspecific reactivities were detected by convEIA or newWB in HCMV-uninfected pregnant women. NewWB would therefore be useful in screening for HCMV infection during pregnancy, allowing subsequent virological tests and prenatal diagnosis in the case of positive results.

We also monitored by newWB a group of 24 transplant recipients with acute HCMV infection within the first year of transplantation. In 32.4% of the patients, the newWB gave a positive signal coinciding with the positive result detected by antigenemia (but later than PCR). In one patient, the positive result by newWB preceded that obtained by antigenemia. These results are in contrast with published data (2, 4, 17) indicating that antigenemia appeared earlier than IgM serology and can be explained by the much higher sensitivity of the newWB with respect to conventional tests. Since newWB always gives a positive result coinciding with the appearance of symptoms, it could be a useful early diagnostic tool to ascertain an HCMV infection after transplantation as a diagnostic parameter complementary to antigenemia.

As expected, the newWB cannot distinguish between symptomatic and asymptomatic HCMV infections. Furthermore, the presence of IgM detected by newWB lasts longer than the symptoms and the positive results detected by antigenemia, and therefore, it cannot be considered a parameter useful for monitoring therapy. Although serological diagnosis in general is less attractive than virological diagnosis because of the immunological disorders occurring in transplant recipients, when performed with a sensitive and specific test, it could be an additional means of obtaining an early diagnosis of ongoing HCMV infection.

Of the sera from transplant recipients and pregnant women, we observed 15 sera that reacted with more than two viral proteins and did not show any reactivity with recombinant proteins. In order to determine whether these sera could represent true IgM-positive sera which did not react with the two recombinant proteins present in the newWB, we tested them with two other recombinant proteins that have been recently described as early gene products highly reacting with HCMV-specific IgM (UL57 [16, 28]). None of the 15 sera gave a positive reaction with the two UL57 recombinant proteins (data not shown), suggesting that ppUL57 is not essential to increasing the sensitivity of the assay.

Studies are under way to compare the newWB with different serological tests for anti-HCMV IgM detection and with EIA kits from different commercial sources in order to determine whether the newWB can be considered a reference test for HCMV-IgM serology. The possibility of transforming the newWB into a commercially available test is under consideration.

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