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# Assessment of Human Cytomegalovirus Antibody Detection Techniques

**Brief Review**

By

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## Introduction

The involvement of human cytomegalovirus (HCMV) in a myriad of ills (53, 89) ranging from congenital malformations (32) to serious, sometimes fatal, complications following transfusion (3, 4, 57) and renal (33, 64) or bone marrow (58) transplantation emphasizes the necessity for diagnostic techniques capable of early and reliable detection of HCMV infection. In addition, epidemiological studies for comparative purposes should employ techniques which yield early, close to 100 per cent detection of CMV antibodies. Such studies should also furnish consistent and reliable information concerning the class of immunoglobulins (Ig's) involved and the formation of antibodies to the various HCMV antigens.

There is almost no serological technique which has not been applied to the diagnosis of HCMV infection (Table 1) (49). However, the use of a variety of techniques has made comparison of data a bit confusing. For instance, in studying pregnant women in an attempt to establish a relationship between time of predelivery HCMV infection and consequences of such infection to offspring, techniques of varying sensitivity have been used. It is difficult to define from such studies when, and if, primary infection represents the greatest risk of congenital malformation. It is also important to detect with certitude positive donors whose blood is destined for multiply transfused patients (4).

For the above reasons and because HCMV infections are coming more to the fore, it seemed an opportune moment to review critically methods capable of measuring the humoral immune response to HCMV and weigh their respective merits and disadvantages.

## Criteria

The criteria for a good test are much the same as for any virus.

a) The test should be broadly reactive with HCMV's but not cross-react with other members of the Herpesviruses.



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Table 1. *Techniques which have been applied to the detection of HCMV antibodies*

| Abbre-viation | Full name | Authors having used the technique in one-way or comparative studies |
|---|---|---|
| NT | Seroneutralization | 1, 5, 6, 21, 29, 63, 74, 86, 87, 90, 91. |
| CF | Complement fixation | 5, 6, 7, 8, 13, 15, 16, 18, 20, 21, 27, 30, 36, 40, 41, 43, 45, 49, 50, 52, 68, 71, 72, 73, 79, 84, 86, 88. |
| IHA | Indirect Hemagglutination | 5, 7, 12, 14, 15, 16, 21, 44, 55, 72, 86. |
| EIA | Enzyme immunoassay | 13, 14, 69, 83. |
| RIA | Radioimmunoassay | 44. |
| IP | Immunoprecipitation | 36. |
| CIE | Counter immunoelectrophoresis | 20. |
| IF ACIF | Immunofluorescence (including anticomplementary immunofluorescence) | 9, 22, 23, 25, 26, 27, 28, 30, 35, 39, 40, 46, 48, 50, 51, 53, 59, 60, 61, 62, 63, 65, 68, 72, 74, 78, 80, 81, 82, 88. |
| IPO | Immunoperoxidase | 26, 27, 47. |

Table 2. *Human Cytomegalovirus-induced antigens as detected by immunofluorescence*

| Designation | Time of appearance | Aspect | Tech-nique | Refer-ences |
|---|---|---|---|---|
| Immediate Early[a] nuclear | 20 minutes | homogeneous diffuse | IIF[c] | 53, 62 |
| CMNA nuclear (early) | 2 hours<br>3 hours | homogeneous diffuse | ACIF[d]<br>ACIF | 25<br>23 |
| Early membrane | 12 hours | | IIF | 9 |
| Early nuclear | 12 hours | diffuse, granular | IIF | 82 |
| Early cytoplasmic | 24 hours | diffuse, granular | IIF | 82 |
| Perinuclear (early) | 24—36 hours | halo around nucleus | IIF | 82, 35 |
| Early nuclear | 24—36 hours | large granules | IIF | 82, 35, 51 |
| Early cytoplasmic | 24—36 hours | large granules | IIF | 82, 35, 51 |
| "Early" nuclear | 72 hours (Ara-c) | diffuse | IIF<br>ACIF | 80<br>28 |
| IgF-Fc receptors | 36 hours | non-specific cytoplasmic | IIF | 22, 39<br>59, 65 |
| Late membrane[b] | 48 hours | | IIF | 9 |
| Late nuclear | 4 days | inclusion body | IIF | 61 |
| Late cytoplasmic | 4 days | diffuse | IIF | 61 |

[a] "Early antigens" = those appearing before viral DNA synthesis
[b] "Late antigens" = those appearing after viral DNA synthesis
[c] Indirect immunofluorescence
[d] Anticomplement immunofluorescence

b) Results should be reproducible both in time and from one laboratory to another. Reproducibility depends in part on the range between negative and positive reactions. If this range is too small, a one or two dilution variation may lead to false negatives.

c) Sensitivity should be great enough to detect small amounts of antibody in dilute samples. Requirements in sensitivity should extend to include differentiating between the various classes of Ig's (G, M, A) and between antibodies against various HCMV antigens (Table 2).

d) Techniques should not require important investments of time or expense. When possible, commercially available reagents should be used, thereby eliminating time consuming preparation and lot-to-lot verification. Results should be rapidly obtained and on a large number of samples at one time.

## HCMV Antigens

The HCMV genome of $147 \times 10^6$ daltons (24, 42) can theoretically code for some 70 to 80 proteins. Any virus-induced or virally modified infected cell protein may be an antigenic element in reagents used in serological reactions.

Before viral DNA synthesis occurs, 10 early polypeptides are made which range in molecular weight from 75 to 19K (76, 77). Only two glycosylated polypeptides appear before viral DNA synthesis. After viral DNA has replicated, some 26 polypeptides can be detected in infected cells, 8 of which are glycosylated. Pulse-chase experiments indicate that none of the early polypeptides is a precursor of those appearing after viral DNA replication (late proteins) (77).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified virions reveals a minimum of 20 (19) and a maximum of 35 (75) bands ranging in molecular weight from 295K to 13.5K (17, 19, 66, 67, 75), of which 8 (31) to 10 (67, 76) are glycoproteins.

Another important constituent of infected cells are dense bodies, characteristic intracytoplasmic accumulations of viral structural proteins. These dense bodies have been shown to contain all (66) or most (19) of the viral structural proteins except one, a polypeptide of 29K.

Very little is known about which polypeptides specific to infected cells, dense bodies and virions are involved in antigen-antibody reactions. Analysis of immunoprecipitates of the immediate early antigen revealed two polypeptides of 76K and 82K (55b). Stinski (76) found that 12 proteins could be precipitated from infected cells with sera from rabbits immunized with purified HCMV and dense bodies and that 7 glycoproteins could be precipitated with sera from rabbits immunized with Triton X-100-extracted glycoproteins from purified virus and dense bodies.

Tween-ether solubilized antigen prepared from infected cells was analysed by Waner (85). Antigenic material sedimented at 5.5S and was composed of polypeptides ranging in molecular weight from 67K to 85K.

The most analysed antigen is that involved in complement fixation (CF) Fiala *et al.* (18) found two peaks of CF activity after filtration of infected cell sonicates through Bio-Gel. In the first peak, CF activity was associated with molecules in excess of $5 \times 10^7$ daltons, while in the second peak, activity corre-

sponded to proteins of 1.5 to $5 \times 10^7$ daltons. SDS-PAGE analysis revealed as many as 15 major bands and up to 30 minor ones in crude CF antigen. After Bio-Gel separation, antigen electrophoresis under denaturing conditions yielded 5 major bands ranging from 60K to 90K. In analysing glycine-buffer extracted CF antigen (CF-GE) Kim *et al.* (43) also found CF activity associated with material $1.5 \times 10^7$ daltons. SDS-PAGE analysis revealed 2 specific polypeptides of 140K and 66K, the latter being glycosylated. The 66K polypeptide appears to be specific to human CMV since it was not found in mouse CMV CF antigen.

A cascade of antigens has been detected by immunofluorescence in HCMV infected cells (Table 2). There are 3 main categories: "immediate early" (54, 62), "early" (80), and "late" antigens (35, 61). Little is known of their function as proteins. The immediate early antigen(s) may correspond to proteins which modify host cell chromatin template activity and structure (37). Early antigens, as originally defined by The *et al.* (80), represent an accumulation of virus induced protein which probably have a wide variety of functions. Late antigens are composed in major part of viral structural proteins since they are, by definition, located within the nuclear inclusion body, the site of virus assembly.

## Techniques

The methods listed in Table 1 fall into three groups depending on the type and location of the antigen(s) used in the reaction. Seroneutralization involves only viral membrane antigens. IHA[1], CF, EIA, CIE, RIA and IP all confront a "global" antigen composed predominantly of virus particles and dense bodies, but also containing infected cell debris. However, these techniques probably involve different series of antigenic proteins since there is a temporal difference in the appearance of the antibodies that each of them detects (8, 81). Lastly, IF and IF-like techniques (ACIF, IPO) (26) provide information on the microscopic location of antigens both in time and space.

The list of techniques in Table 1 may not be exhaustive but does include all techniques which have been used in comparative studies of HCMV antibody detection. Each will be discussed individually and in relation to other techniques with which they have been compared, as information exists in the literature.

## Seroneutralization (NT)

The reaction most influenced by HCMV strain variation is NT as measured by plaque reduction since it is limited to the viral envelope. The original analysis of HCMV strain differences made by Weller *et al.* (90) twenty years ago is still not definitive. Though DNA—DNA hybridization studies among HCMV's (34) reveal 80 per cent homology between strains and comparative polypeptide analysis of 4 HCMV strains reveals a remarkable similarity in protein composition (31), recent neutralization kinetic studies (87, 91) indicate that there are 3 or 4 antigenic groups. However, Waner and Weller (87) caution that "antigenic subgrouping, as with HSV's, may not be possible; CMV strains may reflect a continuous spectrum of a variable antigenic mosaic . . . Until more information becomes

[1] Abbreviations used in this manuscript are explained in Table 1.

available, a working concept of the antigenic complexities of CMV strains might consider a polar relationship between Davis and Ad-196".

For these reasons, NT should be performed using locally isolated virus, or better, the patient's own isolate. The kinetics of seroneutralization is a method of choice for studying antigenic strain variations (91). However, it requires a tissue culture system and is not, therefore, an ideal technique for routine diagnosis.

The use of complement varies among investigators (5, 74, 86, 91). Although complement-independent NT antibodies are present, the addition of complement enhances neutralization and NT antibodies can be detected to a greater dilution.

### *Comparison Between NT and Other Techniques*

NT antibodies arise before CF antibodies and wane after them (1, 6, 72). Both arise simultaneously when infection is due to reactivation of latent virus (72).

Anticomplement immunofluorescence and microneutralization correlated well (74), which is not surprising since both involve complement-mediated reactions. Indirect immunofluorescence (IIF) using whole cells, as opposed to isolated infected cell nuclei, failed to detect two sera having NT antibodies (74). NT has also been compared to membrane fluorescence as a means of determining strain variations (29). Membrane fluorescence revealed greater antigenic variations between AD-169, Davis and C87 than did NT using the same hyperimmune rabbit sera in both tests. NT appears much less sensitive than IHA (21), possibly due to the fact that IHA antibodies rise before NT antibodies (63).

## Complement Fixation (CF)

The CF reaction has several advantages. All necessary reagents are commercially available. Furthermore, using the same procedure a simultaneous search may be made for antibodies to several viruses.

Much has been written about the properties and preparation of CF antigen (5, 6, 86). The relative merits of frozen-thawed (CF-ET) (5) and glycine-buffer (CF-GE) (4, 8, 15, 86) prepared antigens have been weighed. BETTS *et al.* (8) found that, in general, CF-GE detected antibody formation in primary infected renal transplants as early as 3 weeks before CF-FT antigen. CF-GE detected antibody titer rises after seroconversion, whereas CF-FT did not. CREMER *et al.* (15) found that CF-FT detected more rises in antibody titer than did CF-GE antigen, probably because in serially collected sera there were high titers of antibody which went undetected by CF-FT antigen.

CF-GE antigens consist mainly of virus nucleocapsids with small amounts of enveloped particles and disintegrating dense bodies. CF-FT antigen, on the other hand, contains mostly enveloped particles and intact dense bodies with fewer nucleocapsids (40). TEGTMEIER (79) showed that purified dense bodies alone were capable of serving as CF antigen and could detect diagnostically significant rises in CF antibody titers.

CF activity is associated only with infected cells showing extensive cytopathic effects but can be separated from infectious virus by differential centrifugation (6). No correlation was found between infectivity of antigen preparations and CF activity.

CF activity appears to be associated with proteins ranging in molecular weight from 1.5 to $5 \times 10^7$ daltons (41, 43). SDS-PAGE analysis has revealed a large polypeptide of 140K common to 6 strains of HCMV to MCMV (43), as well as 6 major bands ranging in molecular weight from 60K to 90K (18, 43). Kim *et al.* (43) found a 66K polypeptide in HCMV strains which was absent from mouse CMV.

CF antibodies tend to fluctuate and do not persist indefinitely following primary infection (49, 84). Betts *et al.* (8) did not find this true when using CF-GE antigen.

In children undergoing primary infection, CF antibodies appeared only 2 to 7 months after the onset of viremia (72). When the same 8 coded sera were tested by 27 laboratories (45), 8-fold titer differences were found, although there was 90 per cent agreement from one laboratory to another as to seropositivity. One must consider such variation as important in that CF titers could range from 1:4 to 1:512 (49).

*CF Compared to Other Techniques*

It would appear that CF is the least sensitive technique for HCMV antibody detection (13, 15, 21, 27, 30, 50, 88) with the exception of CIE (71) and IP (36).

Immunofluorescence detects antibody formation before complement fixation does (88), which is practically explicable by the early appearance of antibodies among IgA's and IgM's that do not fix complement (68). CF-GE antigen detected antibody appearance in primary infected individuals at about the same time as IF (8). CF is not well suited for the detection of HCMV-specific IgM's, as discussed at length by Cremer *et al.* (16), and is less reliable than immunofluorescence (50) for such detection. The sensitivity of IPO is also greater than that of CF (27). ACIF can be used to test for CF antibodies and is especially appropriate for sera which, due to anticomplementarity, cannot be tested by CF (73).

CF is much less sensitive than IHA. CF appears more sensitive for detecting rises in antibody titers, but IHA is more reliable for detecting the presence of HCMV antibodies (21). This observation may be attributed to the fact that IHA cummulates IgM and IgG antibodies. Bernstein and Stewart (7) found a 100 per cent correlation between CF and IHA positivity, but IHA titers were 5 to 10 fold higher.

Cappel *et al.* (13) found a correlation coefficient of 0.85 for EIA and CF for IgG antibody detection, though fewer sera were positive by CF than by EIA.

CF is even less sensitive than virus isolation in children actively excreting virus (52).

CIE (20, 71) is one of the few techniques which is less sensitive than CF for HCMV antibody detection, although it does detect antibody formation before CF.

## Indirect Hemagglutination (IHA)

IHA has multiple advantages as a technique for detecting HCMV antibodies (7, 21, 86). The range from negative (1:10) to positive (1:20 to 1:5120) is great. A micro-procedure has been developed (21).

In repeated testing of the same sera after freeze-thawing, there was never more than a two-dilution difference in titer (12, 21). IHA can detect IgM, as well as IgG, antibodies, though serum must be plentiful enough to allow gradient separa-

tion of 7S and 19S components. Such detection is little affected by the presence of rheumatoid factor (16). IHA has been shown to be positive when sera contain only IF antibodies to early antigen, therefore indicating that IHA probably detects infection at a very early stage (55a).

IHA is ideally suited to epidemiological surveys (10, 11, 12) It can be performed using blood collected on phenol-keto-urine test cards (12) which can be stored dried for 3 weeks at room temperature and indefinitely at —30° C. IHA can also serve to differentiate virus strains when an unknown isolate is used as competing antigen.

Despite the above advantages IHA poses a problem as concerns the preparation of reagents, notably the sensitizing of sheep red blood cells (SRBC) with virus. Tanning SRBC is a delicate process and requires a stock of antigen prepared in the absence of calf serum. Sensitized SRBC are unstable and must be prepared frequently. Preliminary results obtained in our laboratory (in collaboration with Dr. A. Boué, Château de Longchamp, Paris) demonstrate that sensitized SRBC preserved by lyophilisation are stable for as long as 3 months at 37° C. It is therefore hoped that IHA may be developed into a simple test.

### *IHA Compared to Other Techniques*

With few exceptions all comparative studies between IHA and other methods of HCMV antibody detection have found IHA to be more, or at least equally, sensitive (14, 21, 44, 55a, and see above for comparison of IHA with NT and CF).

Reports are conflicting as concerns the relative sensitivities of IHA and IF. Stagno *et al.* (72) found that IF was more sensitive, though IHA antibodies have a more persistent nature than those involved in IF. On the contrary Michelson *et al.* (55a) found IHA detected 10 per cent more seropositive individuals than did IF.

Castellano *et al.* (14) found that EIA titers were at least 2 to 10 fold higher than IHA titers, but IHA was more sensitive in detecting the presence of antibody.

RIA titers were of the same magnitude as those of IHA (44).

## Enzyme Immuno-Assay (EIA)

Relatively few reports have appeared concerning the use of EIA for detection of HCMV antibodies (13, 14, 69, 83). EIA has the advantage of being very sensitive and of not requiring coverslip preparations, sensitizing of SRBC, or elaborate equipment. It uses inexpensive commercially available peroxidase-labeled anti-IgG and antigens (13) and allows working with very diluted sera. To its disadvantage is the requirement for a tissue culture system for antigen preparation, and high non-specific background levels are a real problem.

EIA holds great promise for the detection of HCMV-specific IgM (13, 69). Cappel *et al.* (13) found they could detect IgM using a commercially available CF antigen without false positives. Schmitz *et al.* (69) found that cytoplasmic contamination of crude antigen preparations caused non-specific binding of conjugate. Furthermore, they obtained false positives when sera contained rheumatoid factor, anti-nuclear IgM, or bacterial contamination. Non-specific binding can almost be eliminated by using as antigen virus in culture supernatant

that is separated from cell debris by centrifugation onto a 60 per cent sucrose cushion (J. PRÉVOT, personal communication).

### *EIA Compared to Other Techniques*

Few comparative studies have been made between EIA and other serological tests (13, 14, 69, 83). Comparison to CF (13) and IHA (14) was discussed above.

EIA compares favorably with immunofluorescence for IgM detection (69) and yields higher titers (83). EIA was improved when commercially available antigen was replaced with isolated infected cell nuclei.

## Radioimmunoassay (RIA)

KNEZ *et al.* (44) are the only authors to have applied RIA to the diagnosis of HCMV infection. Repeated testing of sera for both HCMV-IgG and IgM showed the test to be reproducible within a 2.5-fold range. However, the proportional adjustment of each component appears fastidious. The apparent necessity of adsorbing out IgG with protein A before IgM testing and the use of radioactive iodine makes RIA impracticable in routine laboratory diagnosis.

### *RIA Compared to Other Techniques*

RIA was compared to IHA and CF (44). RIA HCMV-IgG titers were higher than either CF or IHA titers. RIA HCMV-IgM detection, while not compared directly, followed a logical pattern with respect to various in CMV-IgG titers obtained by RIA, CF and IHA.

## Immunoprecipitation (IP)

The use of IP in agar diffusion techniques as a means of diagnosis does not appear to be very practical since it requires enormous amounts of CMV antigen. No relationship could be ascertained between the number of precipitation bands observed and the type of virus used as antigen (36).

### *IP Compared to Other Techniques*

Immunoprecipitation has only been compared to CF (36). Precipitation was negative when CF titers were low.

## Counterimmunoelectrophoresis (CIE)

FORTUNATO *et al.* (20) showed that CIE can be used for HCMV antibody detection if commercially available antigens are carefully screened since very potent antigens are required.

### *CIE Compared to Other Techniques*

Although there was good agreement between CIE and CF, sera with CF titers below 1:128 were negative with CIE. Even highly CF positive sera could not be diluted more than 1:4 (20).

## Immunofluorescence (IF)

IF is a very special technique. It is carried out on a microscopic level and entails very small amounts of antigen. It provides information about CMV antibodies not obtainable by other means and about CMV antigen location in time and space. It can distinguish between antibodies in IgM and IgA which escape detection by complement fixation (68).

Table 1 (see also discussion of HCMV antigens) illustrates the number of HCMV antigens which have been described using IF. Antibodies against IEA correlated very well with active virus excretion (56). The presence of antibodies to EA is indicative of recent infection (80). The presence and/or persistence of anti-EA antibodies is helpful in the differential diagnosis between natally and congenitally acquired infection in children (72).

Unfortunately IF requires a tissue culture system. Anti-LA positivity may be difficult to interpret due to non-specific staining of virus-induced IgG-Fc receptors (22, 39, 59, 65). Various means of circumventing such nonspecific staining have been devised. Direct immunofluorescence appears to give the best resolution (48). Simian virus used to infect human cells (78, 88) does not induce IgG-receptors. Stagno *et al.* (74) performed fluorescence on nuclei isolated from infected cells. In our laboratory (text in preparation), we remove cytoplasm *in situ* from coverslip preparations of infected cells.

HCMV infection in the acute phase of HCMV-induced mononucleosis and in graft recipients is sometimes accompanied by autoimmune phenomena which may lead to false nuclear staining (2, 38). Sera should therefore be tested simultaneously on uninfected cells.

### *IF Compared to Other Techniques*

IPO appears to be less sensitive than IF (27) and gives lower titers. In all instances (30, 60, 74) ACIF and IF gave almost identical results, with ACIF titers being at most 4 times higher (74). Kettering *et al.* (40) report an abbreviated ACIF technique which is no more time-consuming than IF and avoids non-specific staining. Quantitative CF assays done in parallel with ACIF indicate that C' is fixed strongly to late antigens.

Krech *et al.* (46) found a 100 per cent correlation between virus isolation and the presence of IF antibodies, not surprising in that IF antibodies appear before virus can be isolated (63). IF and IPO (26) can complement isolation by showing that observed cytopathic effects are indeed due to HCMV.

## Immunoperoxidase (IPO)

Kurstak *et al.* (47) were the first to apply immunoperoxidase to the study of HCMV. Gerna *et al.* (36) were the first to investigate IPO as a diagnostic tool in identifying isolates and to compare direct and indirect IPO techniques. The direct technique proved superior as it avoids background staining of uninfected cells. Individual infected cells were detected 24 hours p.i.. IPO can be performed in 90 minutes directly on the culturing surface and yields permanent results. However, as yet no IPO-conjugated anti-IgM sera exist on the market.

For comparison with other techniques see above discussions.

## Conclusions

If one were to arrange methods which determine the presence or absence of CMV antibody in order of their sensitivity that order might be as follows:

EIA, RIA, IHA, CF, CIE, IP.

Both EIA and RIA still confront serious problems of background activity and their methodology is not adapted to routine laboratory use. Until such problems are resolved, IHA becomes the next best diagnostic tool. Here again, practical problems exist in the frequent preparation of sensitized red blood cells with antigens prepared in the absence of calf serum and $Ca^{++}$ and $Mg^{++}$ ions. CF has the advantage of using commercially available reagents and is best adaptated to routine diagnosis. However, the decrease in sensitivity of CF compared to that of IHA is the price to be paid for the reduced technical load. CIE and IP combine all the disadvantages mentioned: low sensitivity and requirement for a tissue culture system. In sum, IHA would appear to combine high level of sensitivity with technical practicability.

IF and NT should be considered separately from methods which test just for the presence or absence of HCMV antibodies. NT is of limited diagnostic value in determining the infected state, but is irreplaceable when information concerning viral strain variation is being sought.

IF furnishes a wealth of information. It provides an inventory of the types of antibody being formed and determines whether antibodies appear among G, M or A immunoglobulins. A disadvantage of IF is the requirement of a tissue culture system.

Because HCMV is endemic and associated with a diversity of diseases, tests which merely indicate the presence or absence of antibody are not of sufficient diagnostic value. Information must also be acquired about the formation of specific antibodies in IgM before incriminating HCMV. However, even HCMV-specific IgM may not be indicative of primary infection since they also reappear in seropositive subjects during virus reactivation (70). Further information may therefore be required to establish HCMV as a causal agent.

Any number of techniques (IHA, EIA, CF) can be used for determining the presence or absence of HCMV-specific antibody. A few (IHA, IF, EIA) can reliably define the class of Ig's involved in antibody formation. So far, only one technique, IF, can determine to which group of HCMV antigens antibodies are being formed. Thus, for any study which aims at more than just taking a census of HCMV positive and negative individuals, IF should be used in parallel to provide a maximum of informations.

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