Standardization of the Human Cytomegalovirus Antigenemia Assay by Means of In Vitro-Generated pp65-Positive Peripheral Blood Polymorphonuclear Leukocytes

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We generated in vitro human cytomegalovirus (HCMV) pp65-positive polymorphonuclear leukocytes (PMN) resembling those detected in vivo, following cocultivation of PMN from healthy donors and wild-type HCMV-infected endothelial cells or fibroblasts. After purification, PMN are suitable for preparation of cytopsins which can be used for the antigenemia assay. Cytospin preparations containing a predetermined number of in vitro-generated pp65-positive PMN were used to test some of the major parameters involved in performing the antigenemia assay. The results showed or confirmed that (i) formalin fixation followed by permeabilization is the best fixation procedure developed to date, (ii) the test performance levels provided by different pools of pp65-specific monoclonal antibodies may be significantly different, and (iii) long-term storage (for an unlimited time) is best achieved by keeping fixed slides at -80°C, whereas short-term storage (for up to 1 month) is best achieved by keeping unfixed slides at room temperature. This finding signifies that slides can be shipped all over the world at room temperature. In conclusion, the newly developed procedure for in vitro generation of pp65-positive PMN will provide the basis for standardization of the HCMV antigenemia assay and development of quality control programs.

Since its introduction in 1988 (13, 14), the procedure for antigenemia assay for detection of pp65 of human cytomegalovirus (HCMV) in polymorphonuclear leukocytes (PMN) has undergone multiple modifications and improvements of cell fixation, immunostaining procedure, and use of pooled monoclonal antibodies (3). However, standardization has not been achieved thus far, and results obtained in different laboratories are difficult to compare. The major obstacle to the standardization of the antigenemia assay has been the lack of biological material (pp65-positive PMN) available in unlimited quantities and standardized with a predetermined ratio of pp65-positive PMN/pp65-negative PMN. Thus far, the only positive control introduced into a commercially available kit for determination of pp65-antigenemia (5) consisted of cytospin preparations containing insect cells expressing HCMV pp65 mixed with PMN from healthy volunteers (12).

In the present report, we describe the use of the recently developed procedure for generating in vitro pp65-positive PMN (9) for the optimization and standardization of the HCMV antigenemia assay. The availability of a proper positive control can lead to development of standardization protocols and quality control programs in the near future.

MATERIALS AND METHODS

Isolation of PMN. Concentrated preparations of human PMN from either HCMV-seropositive or HCMV-seronegative healthy donors were obtained as follows. One milliliter of 6% dextran solution (molecular weight, 70,000) in HCMV-seropositive or HCMV-seronegative healthy donors were obtained as 0.0095-1137/98/$04.00 © 1998, American Society for Microbiology. All Rights Reserved.

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2 × 10⁵ PMN used for preparation of each cytospot, and (ii) the degree (intensity) of staining, which was determined by classifying positive cells into the three staining categories of weak (1+), moderate (2+ or 3+), and strong (4+).

**Statistical analysis.** Comparison between means was performed with the Student t test, whereas the difference in distribution of staining categories was analyzed by the Pearson chi-square test.

**RESULTS**

In vitro generation of pp65-positive PMN. Initial attempts at generating pp65-positive PMN following coculture with HELF infected with the reference HCMV strain AD169 were consistently unsuccessful. Similarly negative results were obtained when other laboratory-adapted strains, such as Davis or Towne, were used. pp65-positive PMN were generated when PMN were cocultured with HUVEC infected with a clinical HCMV isolate from blood (VR6110) (Fig. 1). Similar results were also achieved with HELF infected with different isolates from different body sites of both immunocompromised subjects and immunocompetent subjects. No pp65-positive PMN were observed when contact of PMN with infected cells was prevented by using Transwell inserts (0.4-μm-pore-size membranes) or when contact was restricted to infectious cell-free wild-type virus.

**PMN purification and testing.** Once generated, pp65-positive PMN had to be separated from contaminating HCMV-infected cells. Since the level of purification achieved after Transwell insert migration was not substantially increased by the additional FACS purification, Transwell migration was subsequently used as a routine purification step. Then, serial concentrations of pp65-positive PMN were obtained in cytospots set up following preparation of serial mixtures, at a different proportion, of a known pp65-positive migrated PMN suspension and a PMN suspension from healthy donors. Results of testing of replicate cytospin preparations containing a different number of pp65-positive PMN are reported in Table 1, where the coefficient of variability of the mean is consistently below 10% when levels of antigenemia are >50, while it becomes progressively higher with antigenemia levels approaching 1.

**Fixation.** The better performance of the formalin plus NP-40 fixation over the methanol-acetone method was confirmed in terms of both number of pp65-positive PMN and staining intensity (data not shown). In addition, some experiments were carried out to verify whether NP-40 permeabilization of PMN previously fixed with formalin was a critical step. It was found that, although the fluorescent staining was more evenly distributed over the entire nucleus of positive cells in permeabilized PMN preparations, both the number of positive cells and the degree of staining were not significantly different in the two groups of cytospin preparations (data not shown).

**Immunostaining technique.** No significant differences were found among IFA, IPA, and ABC (Table 2). In addition, although the distributions of different PMN staining groups were not significantly different among the three tested techniques, the absolute number of PMN belonging to the weak IFA staining group was greater than the relevant IPA and ABC group values, whereas the number of strongly stained cells was slightly lower for the IFA technique.

**Pool of monoclonal antibodies.** The results of the comparison between the sensitivity of our pool (referred to as PV pool)
TABLE 2. Sensitivities of different immunostaining techniques with formalin plus NP-40 as fixative (prior to storage at −80°C) and the PV pool

<table>
<thead>
<tr>
<th>Staining technique (No. of replicates)</th>
<th>Median no. of pp65-positive PMN (range)</th>
<th>P valuea</th>
<th>Median no. (%) of pp65-positive PMN with degree of staining</th>
<th>P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>IFA (10)</td>
<td>205.5 (160–240)</td>
<td></td>
<td>51.5 (25)</td>
<td></td>
</tr>
<tr>
<td>IPA (10)</td>
<td>200 (145–230)</td>
<td>NSc</td>
<td>130.5 (64)</td>
<td></td>
</tr>
<tr>
<td>ABC (10)</td>
<td>203 (148–245)</td>
<td>NSc</td>
<td>23.5 (11)</td>
<td></td>
</tr>
</tbody>
</table>

* For each pool, data for 10 replicates are shown.

a t test.

b Pearson chi-square.

c NS, not significant.

TABLE 3. Sensitivities of different pools of HCMV pp65-specific monoclonal antibodies with formalin plus NP-40 as fixative (prior to storage at −80°C) and IFA as immunostaining technique

<table>
<thead>
<tr>
<th>Monoclonal antibody pool (no. of monoclonal antibodies)</th>
<th>Median no. of pp65-positive PMN (range)</th>
<th>P valueb</th>
<th>Median no. (%) of pp65-positive PMN with degree of staining</th>
<th>P valuee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>PV pool (3)</td>
<td>205.5 (160–240)</td>
<td>0.0041</td>
<td>51.5 (25)</td>
<td>0.0047</td>
</tr>
<tr>
<td>Argene (2)</td>
<td>169 (140–210)</td>
<td></td>
<td>130.5 (64)</td>
<td></td>
</tr>
<tr>
<td>Biotest (2)</td>
<td>138 (117–153)</td>
<td>&lt;0.00001</td>
<td>23.5 (11)</td>
<td></td>
</tr>
<tr>
<td>Chemicon (2)</td>
<td>152.5 (110–179)</td>
<td>0.0008</td>
<td>64 (38)</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>87.5 (52)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.5 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

* For each pool, data for 10 replicates are shown.

† t test.

‡ Pearson chi-square.

§ NS, not significant.
for short-term staining or for long-term storage at −80°C following fixation upon delivery.

Using in vitro-generated pp65-positive PMN, we have confirmed that fixation with formalin provides better results than fixation methods involving acetone, as previously reported by our group as well as by others (1, 3, 8). The use of the formalin fixation procedure without the subsequent permeabilization step has recently been recommended because of its simplicity (8). On the basis of this study, we believe that, although the suppression of the permeabilization step does not cause a significant decrease in terms of absolute number of positive cells, a more even distribution of specific staining is obtained in permeabilized cells, while the time required for test performance is only slightly longer (5 min).

As for the immunostaining technique, we previously reported significantly better results given by the IFA compared to the IPA or ABC procedure (3). We attributed this difference to the procedure incorporating use of methanol-H$_2$O$_2$ for removal of endogenous peroxidase activity. However, when 3-amino-9-ethylcarbazole in acetate buffer was used for detection of enzymatic activity (13, 15), no significant background staining was encountered and no difference in terms of positive cells was noted. In fact, no differences among IFA, IPA, and ABC were found in this study.

All three commercially available pools of pp65-specific monoclonal antibodies tested in this study were significantly less sensitive than the PV pool. However, the Argene pool was found to possess a significantly higher sensitivity than the Biotest pool, as already reported (2), whereas no significant difference was observed between the Argene and Chemicon or between the Chemicon and Biotest pools. We believe that from this point on, use of in vitro-generated pp65-positive PMN reference preparations will enable testing of new pools of monoclonal antibodies and evaluation of their actual test performance.

One of the major objectives of this study was to comparatively evaluate the optimal storage temperatures of fixed and unfixed slides. It was shown that unfixed slides are best stored at room temperature for up to 1 month, while it was already known that fixed slides are best stored at −80°C for unlimited periods. The optimal storage of unfixed slides at room temperature for a reasonable period would allow shipment at room temperature of cytospots to all over the world.

The newly developed procedure for in vitro preparation of cytospots finally allows us to address the problem of long-term storage of large batches of slides containing a predetermined number of pp65-positive PMN. In fact, thus far, only the sensitivity of the antigenemia assay for cytospin preparations from freshly collected blood samples has been compared to the sensitivity achieved for preparations from blood samples stored at room temperature or at 4°C for 24 to 72 h. In this respect, several reports, although unanimously recognizing a progressive loss in sensitivity of the antigenemia assay during storage at room temperature, have failed to quantitatively evaluate the effect of storage temperature on the antigenemia assay sensitivity.

### Table 4. Effect of temperature during storage for a 7-day period on fixed and unfixed PMN slides with IFA and the PV pool

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>Fixation upon slide preparation</th>
<th>No. of replicates</th>
<th>Median no. of pp65-positive PMN (range)</th>
<th>$P$ value$^a$</th>
<th>Median no. (%) of pp65-positive PMN with degree of staining</th>
<th>$P$ value$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT$^a$</td>
<td>None</td>
<td>5</td>
<td>178.0 (160–196)</td>
<td>0.0009</td>
<td>30 (17) (133 (75) 15 (8))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formalin + NP-40</td>
<td>5</td>
<td>109 (86–117)</td>
<td></td>
<td>34 (31) (70 (64) 5 (5))</td>
<td>0.012</td>
</tr>
<tr>
<td>−80°C</td>
<td>None</td>
<td>5</td>
<td>129 (114–144)</td>
<td>0.004</td>
<td>0.029 (38 (29) 87.5 (68) 3.5 (3))</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Formalin + NP-40</td>
<td>6</td>
<td>157.5 (134–192)</td>
<td>0.0186</td>
<td>38.5 (24) (99 (63) 20 (13))</td>
<td>0.005</td>
</tr>
</tbody>
</table>

$^a$ RT, room temperature.
$^b$ $t$ test.
$^c$ Pearson chi-square ($P = 0.0016$, overall comparison of three degrees of staining at different temperatures).
$^d$ NS, not significant.

### Table 5. Long-term effect of storage at room temperature of unfixed cytospots on HCMV antigenemia assay sensitivity with the PV pool

<table>
<thead>
<tr>
<th>Duration of storage at room temperature</th>
<th>No. of replicates</th>
<th>Median no. of pp65-positive PMN (range)</th>
<th>$P$ value$^a$</th>
<th>Median no. (%) of pp65-positive PMN with degree of staining</th>
<th>$P$ value$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>6</td>
<td>69.5 (58–76)</td>
<td></td>
<td>15 (21) (48 (69) 6.5 (10))</td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>69 (58–81)</td>
<td></td>
<td>15 (22) (47 (68) 7 (10))</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td>8</td>
<td>67 (59–73)</td>
<td>NS</td>
<td>15 (22) (45 (67) 7 (11))</td>
<td>0.005</td>
</tr>
<tr>
<td>30 days</td>
<td>5</td>
<td>70 (53–91)</td>
<td>NS</td>
<td>26 (37) (38 (54) 6 (9))</td>
<td>0.003</td>
</tr>
<tr>
<td>40 days</td>
<td>5</td>
<td>45 (43–47)</td>
<td>0.0015</td>
<td>15 (34) (27 (60) 3 (7))</td>
<td></td>
</tr>
<tr>
<td>55 days</td>
<td>5</td>
<td>26 (20–28)</td>
<td>0.0005</td>
<td>12 (46) (12 (46) 2 (8))</td>
<td></td>
</tr>
<tr>
<td>75 days</td>
<td>5</td>
<td>1 (0–5)</td>
<td>0.0001</td>
<td>1 (100) (0 (0) 0 (0))</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $t$ test.
$^b$ NS, not significant.
storage, have provided somewhat controversial findings (1, 6, 7, 10).

In conclusion, we believe that standardization protocols and quality control programs may be developed for the HCMV antigenemia assay by taking advantage of the novel procedure for generating pp65-positive PMN in vitro.

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