Balance between herpes viruses and immunosuppression after lung transplantation

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Chapter 2

Towards standardization of the human Cytomegalovirus Antigenemia assay

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Abstract

The Human Cytomegalovirus antigenemia (HCMV-Agemia) test has been accepted worldwide as a clinical tool in the diagnosis and management of HCMV-associated syndromes in immuno-compromised patients. The many modifications proposed since the first description by our laboratory make standardisation of the HCMV-Agemia assay necessary to enable multicentre clinical trials. We report the initial work for standardization of the HCMV-Agemia assay. A standard protocol is proposed, the optimal distribution conditions are investigated and the results of the shipment of positive and negative test slides as well as of two sets of coded internal standard slides are discussed. The main conclusions are that standard slides can be distributed at room temperature and that the results of participating laboratories with the coded internal standard slides were strikingly similar in spite of differences in HCMV-Agemia protocols used by participating laboratories.

Introduction

Since the Human Cytomegalovirus (HCMV) antigenemia assay (HCMV-Agemia assay) was first described by our laboratory more than 10 years ago (1), numerous studies have confirmed its clinical relevance for the diagnosis of active human HCMV-infection. Its high diagnostic accuracy, rapidity and technical simplicity have made the HCMV-Agemia assay one of the cornerstone methods for the diagnosis and management of active HCMV infection in immune compromised patient (2-6).

However, a considerable number of modifications concerning every step in the protocol have been introduced by different laboratories, e.g. the isolation method of polymorphonuclear cells (PMNs), the cell numbers used for preparing slides, the use of cytocentrifuged cell preparations versus methanol spreading, the use of different fixation methods, of different monoclonal antibodies, of chromogenic versus fluorescent detection of positive cells and the reading of the results by only counting the positive cells per slide versus the scoring of positive cells per 50,000 PMNs (7-12). Introduction of these modifications was intended to improve performance of the original assay, but this may lead to increased interlaboratory variation as well. These variations in methodology make comparison of published results difficult and form an obstacle to (future) multicentre trials of antigenemia-directed intervention studies in immune compromised patients with a high risk for fatal HCMV disease. Consequently, a concerted action was initiated to set up a standardization program for the HCMV-Agemia test in several collaborating laboratories and clinical centres in the European Community (13). The primary goal was to come to similar test performances in participating laboratories.

First step was to make recommendations for a standard protocol and distribute it to the participating centres for modifying their current assay or adopting the standard protocol. Since participating laboratories were reluctant to abandon their local modifications, an alternative goal was set. This became the comparison of the HCMV-Agemia test performances in the participating laboratories on identical control slides. Only then could the decision be taken whether a standardized HCMV-Agemia assay is justified and required for multicentre studies. The second step was the evaluation of the performance of the reference laboratory to see whether the reproducibility of the control slides was adequate.

The third step was the investigation of optimal distribution conditions. Effects of temperature and fixation method were analyzed and subsequently

positive/negative control slides were distributed to be tested in the participating laboratories. The last step discussed in this article is the evaluation of two sets of coded slides to evaluate their usefulness as an internal standard. One set of internal standard slides was made of pp65-positive baculovirus-transfected insect cells. A second set comprised pp65-positive polymorphonuclear cells (PMNs) prepared by cocultivation of normal donor PMNs with productively infected endothelial cells (14;15). In the near future, the interlaboratory variation will be evaluated by distributing control slides made of patient material (external standard) with unknown numbers of positive cells.

Materials and Methods

Isolation of leukocytes

Two millilitres of EDTA-treated blood was mixed with 1 ml PBS and 1 ml of a 5% dextran (MW 250,000) in 0.9% NaCl and allowed to settle at 1 g sedimentation force at 37°C at a 60° angle for 10 min. The leukocyte supernatant was collected and centrifuged at 300 *g* for 2 min. The erythrocytes were lysed by resuspending the cell pellet in 2 ml cold erythrocyte lysing buffer (NH_4CL 155mmol/l, $KHCO_3$ 10 mmol/l, $Na_2.EDTA.3H_2O$ 0.1 mmol/l, pH 7.4) at 4°C for 10 min. The cells were washed twice in PBS and then resuspended in PBS and counted. From this a cell suspension of 1.5 x 10^6 cells/ml was made.

Preparation of cytospin/slides

Cytocentrifuge preparations were made using 100 µl cell suspensions (input 1.5 x 10^5 cells) centrifuged at 550 rpm during 5 min (Cytospin 3; Shandon Southern products, Astmoor, United Kingdom). The slides were dried for 15-20 min with a cold blower, wrapped in aluminium foil and stored at -80°C until use.

Fixation and staining procedure

Cells were fixed with formaldehyde and permeabilized with Nonidet P-40 solution as described before (11). Slides were incubated in duplicate with 50 µl 1:5 diluted anti-HCMV- pp65 (C10/C11) (3) for 30 min in a 37°C humid chamber, washed twice with PBS, and subsequently incubated with a 50 µl HRP conjugated goat Fab anti-mouse IgG (H+L) absorbed with human serum (Protos Immunoresearch, Burlingame, Calif., USA) per spot in a 37°C humid chamber for 30 min. After two washings in PBS, the enzyme reaction was performed for15 min with a 3-amino-9-ethylcarbazole (AEC) solution in 0.1 *M* acetate buffer (pH 4.9)

(AEC 10 mg Sigma chemical Co, St Louis, Mo., USA) dissolved in 4 ml *N,N*-dimethylformamide and then filled up to 80 ml with acetate buffer, filtered and supplemented with 75 μl H_2O_2 (30% v/v). The slides were then washed with acetate buffer for 10 min and counterstained with hematoxilin Mayer solution (see below for counting all cells), carefully rinsed with tap water and mounted in Kayser glycerine gelatine (Merck 9242).

Quantification of stained slides

All PMNs with a yellowish/brown nuclear staining were considered positive. Results were expressed as positive cells per 50,000 by counting all cells using a grid (1) or a semi-automated image analyser (Quantimet 500, Leica) (13).

Storage test

Fresh blood samples were taken from 9 solid-organ transplant patients positive in the diagnostic HCMV-Agemia assay, with scores between 10 and 250 per 50,000 PMNs. Six patients had HCMV-Agemia levels between 10 and 50 per 50,000 PMNs, and 3 scored between 50 and 250 per 50,000 PMN. The fixed and unfixed slides of these patients were vacuum sealed and stored in duplicate at room temperature and at -80°C, except for one slide of each patient which was stained on the day of sampling. Frozen slides were brought to room temperature with a cold blower before opening the vacuum seal to prevent the formation of condensation on the slides. Staining (and fixation if necessary) was done at days 1, 2, 7, 14 and 21 according to the standard protocol.

Positive negative slides

All standard slides were made on the day of blood drawing. Negative cytospots were made with PMN from a normal donor. HCMV-pp65-positive insect cells (Baculovirus expressed) were made as described (13) and mixed with normal donor PMN at a ratio to obtain a score of >100. All slides were dried with a cold blower, wrapped in aluminium foil, vacuum-sealed and stored at -80°C until distribution at room temperature (RT).

Preparation of internal standard

For the preparation of the reference standard containing pp65-positive cells, two methods were used. Either HCMV-pp65-positive insect cells (Baculovirus expressed) (13) or pp65-positive cells made by coculture of donor PMNs with HCMV-infected endothelial cells (16) were used. To determine the percentage of positive cells in this cellular mixture, a cytospin slide was made and stained according to the standard protocol, and the pp65-positive cells were

counted. Then the cellular mixture containing the pp65-positive cells was mixed at different ratios with normal donor PMN and cytospins were made (input 1.5 x 10^5 cells). Ratios between pp65-positive cells and normal PMN were chosen to obtain slides with 0, 1-5, 6-10, 25-50, and >100 pp65-positive cells per 50,000 cells. Slides were dried with a blower, wrapped in aluminium foil vacuum-sealed and stored at -80°C until use or distribution. A set of internal standard slides contained 5 coded slides with 2 cytospots each. Six cytospots of each preparation were stained at the reference laboratory to determine the actual number pp65-positive cells per 50,000 PMNs. Mailing by courier delivery to the participating laboratories took place at RT, advice was given to stain the slides upon arrival or to store them at -80°C until use. Participating laboratories received two sets of coded slides A-E marked with the day of preparation without any further information.

Results

Variations in the HCMV-Agemia protocols used by participating laboratories

The fixation method of most (5/8) laboratories was formaldehyde/NP-40, 2 centres used acetone and 1 laboratory used methanol/acetone. The monoclonal antibody against the HCMV-pp65 antigen was a pool of C10/C11 (Biotest Ag, Germany, IQ Products , the Netherlands) in 3 centres and 5 centres used an in-house monoclonal (or a pool of in-house monoclonals) 1 centre used a mixture of an in-house monoclonal and Cinapool (Argene Biosoft) and one centre CINApool only (Table 1).

Storage test

Results of experiments on the influence of fixation and temperature on the quality of the slides evaluated at different time points after storage are shown in Fig 1. No detectable loss of signal even after 3 weeks was noticed in any of the slides kept unfixed at -80°C (Fig. 1D). Remarkably a decrease of the scores was seen in a minority of the slides fixed with paraformaldehyde and subsequently stored at -80°C (Fig. 1C). Similarly fixed slides lost their positivity when stored at room temperature (Fig. 1A). When stored unfixed at room temperature (Fig 1B), no significant loss of signal was observed within the first 2 days of storage and the decline appeared less than with fixed slides stored at room temperature.

Table 1 *Variations in the HCMV-antigenemia protocols in the 10 participating laboratories.*

Fixation method	MoAb	Staining	Quantification
PFA/NP-40	C10/C11(3)	PO	per 50,000 PMN
PFA/NP-40	BM 222[1]	PO	per 50,000 PMN
PFA/NP-40	1C3, 2A6 and 4C1(11)	IF	per 50,000 PMN
PFA/NP-40	C10/C11(3)	IF	per 50,000 PMN
PFA/NP-40	CINApool[2]	IF	per spot
PFA/NP-40	C10/C11(3)	IF	per spot
PFA/NP-40	Clone 10[3]	IF	per spot
Acetone	28/77[1]	PO	per spot
Acetone	pp65-33[1]	PO	per spot
Methanol/Acetone	CINApool + Emmanuel pool[4]	IF	per spot

Table 1 : Data are given as stated by participating laboratories. PFA/NP-40= paraformaldehyde/Nonidet P-40. References of monoclonal used are given when available.
[1] Reference not available
[2] Argene Biosoft cod. 11-002.
[3] Clone 10: developed by Dr. J. Booth
[4]Emmanuel pool: pool of monoclonal antibodies developed by Dr. Emmanuel

Distribution of positive/negative slides

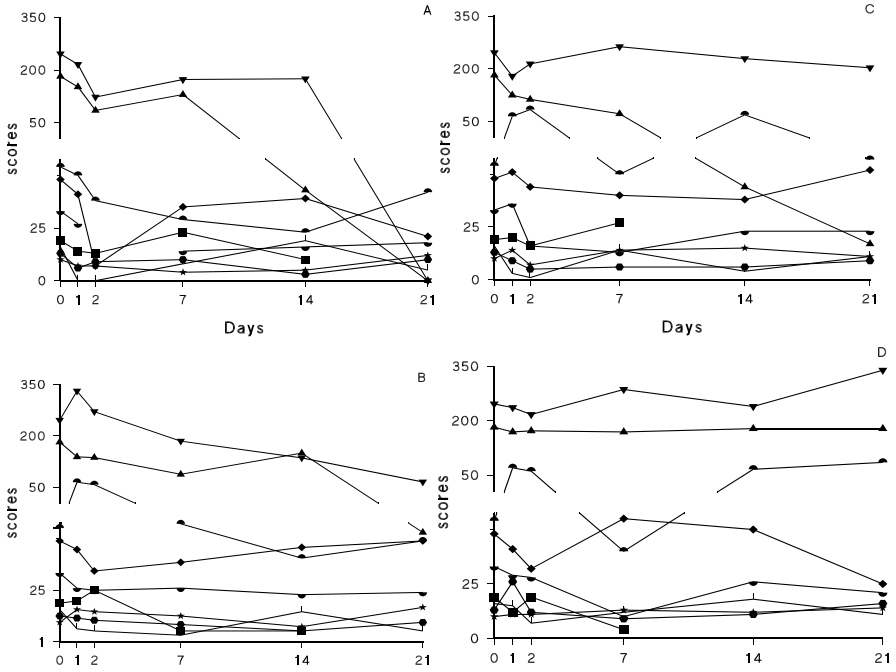
Based on the results described in the previous section, unfixed slides were aluminium wrapped, vacuum sealed and distributed at RT by courier delivery to ensure delivery within 2 days. All participating laboratories reported positive staining of baculovirus transfected HCMV-pp65 pos cells (data not shown). In general cell morphology was maintained well. No false-positive staining was reported by any laboratory.

Internal standard slides

In general, the participating laboratories were satisfied with the overall quality of the internal standard slides without problems with the staining procedure (Fig. 2A,B).

Data are shown in table 2. Average scores of the slides by our laboratory showed that the internal standard preparations made with the Baculovirus system were 0, 6, 8, 61 and 511.This was close to the objected number of pp65-positive cells per 50,000 PMN (0, 1-5, 6-10, 25-50 and >100).

Figure 1 *Influence of fixation, storage temperature and time on slides of 9 CMV-Agemia-test-positive patients. All slides were wrapped in aluminium and vacuum- sealed. Each line represents the scores of one sample.* ***A****) Fixed slides stored at RT.* ***B****) Unfixed slides stored at RT.* ***C****) Fixed slides stored at -80°C.* ***D****) Unfixed slides stored at -80°C.*



The scores of our laboratory with the internal standard slides made with pp65-positive PMN were all within objected ratios (average scores 0, 1, 9, 39 and 680). Seven out of nine participating laboratories returned their data of the coded internal standard slides. No false-positive results were reported. In general, all laboratories were able to detect the pp65-positive spiked cells on the cytospots. With the exception of centre 2, all centres were able to detect an increasing number of pp65-positive cells with an increased number of spiked cells with both types of internal standard slides (Table 2). There was a good correlation between the results of the different laboratories for each slide (Fig 3).

The absolute scores of the slides with 0, 1-5, 6-10, 25-50 and >100 pp65-pos insect cells (baculovirus expressed) showed that, of the centres that quantified per 50,000 PMNs, centre 3 (average scores, respectively: 0, 13, 29, 123 and approximately 1450) scored higher than the reference centre (centre 1).

Table 2 *Results of the 8 responding laboratories of the coded unfixed internal standard slides distributed at room temperature to the 10 participating laboratories*

Centre	number of HCMV-pp65-positive cells per 50,000 or per Cytospot*									
	baculovirus expressed pp65-positive insect cells					pp65-positive PMN made by co-culture				
	0	1-5	6-10	25-50	>100	0	1-5	6-10	25-50	>100
1 (ref)	0	6±2	8±5	61±13	511±141	0	1±1	9±3	39±5	680±106
2	0/0	7/3	0/0	17/55	295/584	0/0	0/0	17/26	5/2	199/142
3	0/0	10/16	22/35	-/123	~1500/~1400	0/0	1/2	8/13	83/75	~670/900
1[1]	0	8±3	10±5	66±13	376±74	0	3±2	22±16	59±10	1079±74
4[1]	0/0	13/8	13/11	114/102	890/677	0/0	0/2	4/8	14/44	596/399
5[1]	0/0	0/7	17/15	44/19	646/420	0/0	1/0	14/14	57/55	1468/2132
6[1]	0/0	19/15	35/27	107/127	ND/ND	0/0	0/0	4/8	26/27	>200/>200
7[1]	0/0	15/13	23/24	124/120	~1200/~1100	0/0	0/0	7/9	37/32	~1100/1000
8[1]	0/0	5/2	22/12	56/37	1000/1000	0/0	3/1	10/11	60/48	700/750

Table 2 : Staining was done according to the local protocol of the participating centre and scores of two cytospots (spot A/spot B) are shown. Results are expressed as pp65-positive cells per 50,000 PMNs or pp65-positive cells per cytospot*. Two kinds of pp65-positive cells were used: baculovirus-expressed pp65-positive insect cells and: in vitro generated pp65-positive PMNs. Results of the reference centre are expressed as mean ±standard deviation (n=6) of both pp65-positive cells per 50,000 PMN and positive cells per cytospot (ND=not done)

[1] Counts performed by cytospot

Figure 3 *Average results of all participating laboratories with the distributed internal standard slides (Table 2). Results are expressed as pp65-positive cells per 50,000 PMNs if available. For centres that quantified per cytospot results per cytospot were used.*

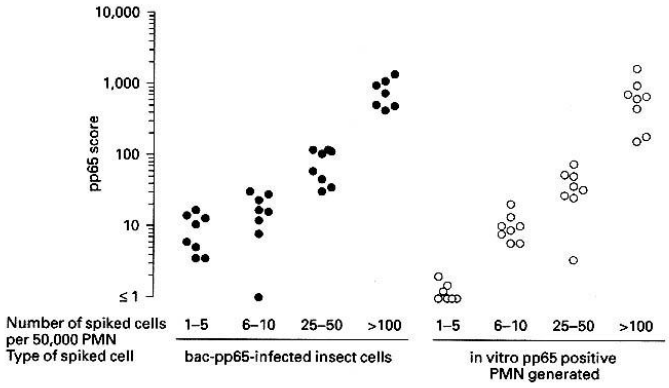
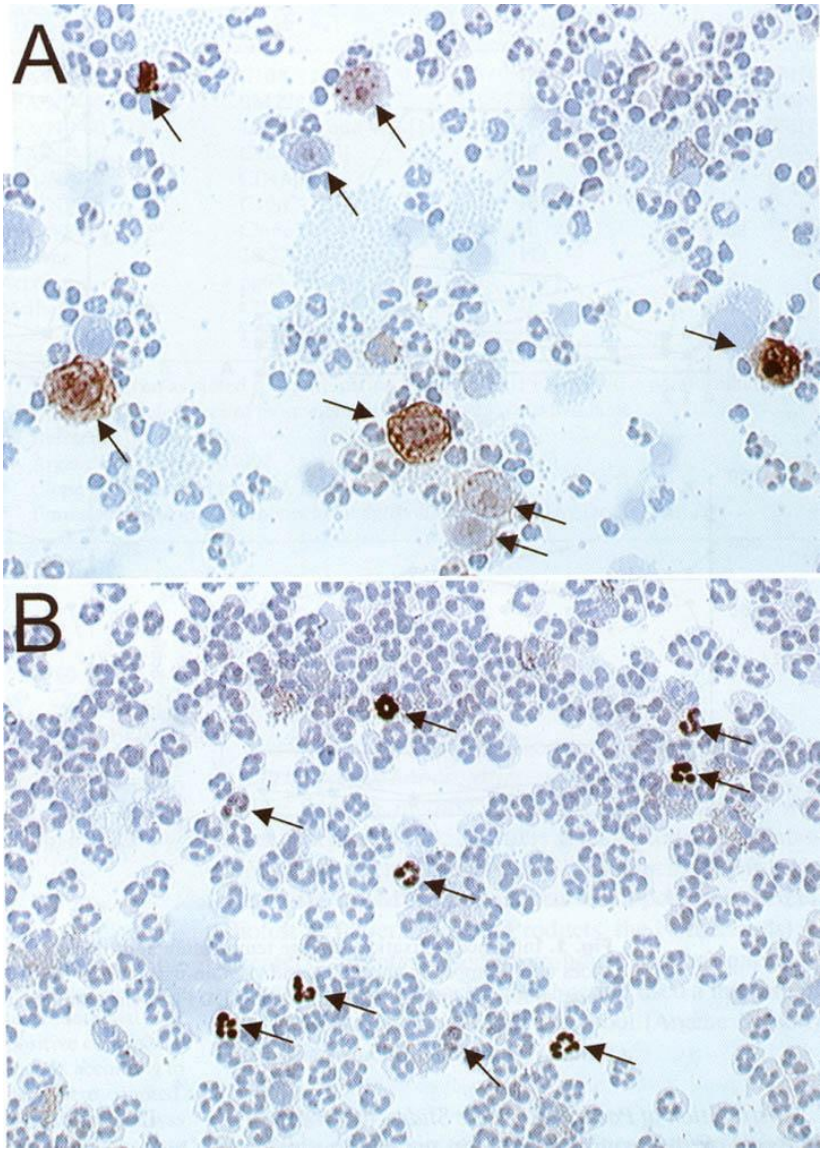


Figure 2 *Photomicrographs of immunoperoxidase stained internal standard slides from the category of >100 pp65-positive cells mixed per 50,000 PMN.* ***A*** *Internal standard slide with pp65 baculovirus-infected insect cells* ***B*** *Internal standard slide with pp65-positive PMNs made by coculture of normal donor PMNs with HCMV infected endothelial cells. Arrows indicate positively stained cells. Note the larger variation in both morphology and staining intensity in the slides spiked with pp65 baculovirus infected insect cells (**A**) as compared to the slide spiked with pp65-positive PMNs.*



The results of centre 3 with the pp65-positive PMN made by coculture of donor PMN with HCMV-infected endothelial cells (average scores of 0, 2, 11, 79 and 785) were at the same level as the results of the reference centre.

The results of centre 2 (average scores 0, 5, 0, 36 and 440 for the pp65-positive insect cells and 0, 0, 22, 4 and 172 for the pp65-positive PMNs) were neither in line with the expected results no with the results of the other participants for both the internal standard slides made with the pp65-positive insect cells as well as the pp65-positive PMNs.

Of the centres that quantified per cytospot centre 5 (average readings 0, 4, 16, 32 and 533 per cytospot) and centre 8 (average readings 0, 3, 16, 47 and 1000 per cytospot) were comparable with the reference centre (average readings 0, 8, 10, 66, 376 respectively per cytospot) and centre 4 (average readings 0, 11, 12, 108, 784 per cytospot), centre 6 (0, 17, 31, 117 and not done per cytospot) and centre 7 (0, 14, 24, 122 and approximately 1150 per cytospot) scored higher in the Baculovirus transfected pp65-positive cells than the reference centre.

With the pp65-positive PMNs, centre 5 (readings of 0, 1, 14, 56 and 1800 per cytospot) and centre 8 (Readings of 0, 2, 11, 54 and 725 per cytospot) had comparable results with those of the reference centre (readings 0, 3, 22, 59 and 1079 per cytospot), whereas centre 4 (readings 0, 1, 6, 29 and 498 per cytospot), centre 6 (readings of 0, 0, 6, 27 and >200 per cytospot) and centre 7 (readings of 0, 0, 8, 35 and 725 per cytospot) had a lower result than the reference centre. However, since these readings were not quantified per 50,000 PMNs, these results may reflect different cell numbers per spot. The ratios between the preparations with 6-10 and 25-50 pp65-positive cells were very similar (3-5) for the pp65-positive PMN in all laboratories that quantified per cytospot. The ratios were less similar (3-9) for the same centres when the Baculovirus system was used. One centre withdrew from the study, one centre could not interpret the results due to high background staining.

Discussion

This study describes the preparation of pp65-positive standard slides and the evaluation of these slides by 8 different laboratories. Although differences in the absolute values were recognized, the results of the readings by the different laboratories were remarkably similar, despite distribution at RT and the differences in HCMV-Agemia protocols used by the participating laboratories. This was especially true for the in-vitro-generated pp65-positive PMNs made by coculture, for which their potential usefulness for standardization was recently described by Gerna et al (16). The main conclusion is that these standard slides

are well suited for standardization purposes and can be distributed at RT, provided they are wrapped in aluminium, vacuum-sealed, and stored at -80°C upon arrival until staining. This enables the different laboratories to evaluate and compare their own assay protocols including differences in fixation techniques. Since the participating laboratories were reluctant to abandon their own modifications of the protocol, it is of major importance to have an internal standard that performs well in all these different laboratories.

Optimal storage conditions were evaluated to determine the distribution conditions. These proved to be storage of unfixed slides at -80°C. Because of the logistic problems involved with distribution of material at -80°C and as no apparent decrease in signal was seen with unfixed slides at RT, we concluded that, for standardization purposes, unfixed slides can be sent at RT. This was confirmed by the distribution of the positive negative slides and later by the results of the internal standard slides.

Distribution and evaluation of these internal standard slides showed remarkable results. None of the responding laboratories had false- positive staining. Six out of 8 responding laboratories detected not only the positive cells but also detected the increasing number of spiked cells in the coded slides. This means that in spite of the differences in the protocols the results are remarkably similar.

At the start of this concerted action only the baculovirus system was available to make pp65-positive cells. Therefore, this system was chosen to prepare the pp65-positive cells for the positive negative slides of the internal standard. However, during the study it became possible to make pp65-positive PMN in vitro (14, 15). Both methods were used to make internal standard slides. Besides morphological considerations, both types of internal standards have two important differences. The pp65 protein is actively produced in the pp65-baculovirus infected insect cells. During the course of infection, the polyhedrin promoter that drives the expression of pp65 is switched on, with maximal expression reached at late stages of baculovirus infection. As baculovirus infections generally are not synchronous, cells will harbor differing amounts of intracellular pp65. This was observed by the participants as differences in staining intensity of the baculovirus-spiked internal standards (Fig. 2A). Thus the criterium for positivity appeared more difficult to set as compared to pp65-positive PMN and probably has led to more variation in the quantitation results (Table 2).

Furthermore, bac-pp65 is a recombinant protein which might influence the staining capacity of some of the anti-pp65 monoclonal antibodies. The use of in-vitro- generated pp65-positive PMN as internal standard may more closely

resemble the patient situation. Nevertheless, these pp65-positive PMN are also a phenomenon generated in vitro in which the pp65 was acquired by co-cultivation of PMN with HCMV-infected endothelial cells. In contrast to infected cells, these PMN do not produce pp65 (17). As it appears, PMNs generally seem to acquire similar amounts of pp65 upon cocultivation, which is reflected in a more homogeneous staining pattern of the pp65-PMN internal standard (Fig. 2B). This allowed for a lower variation in the quantification of this type of internal standard, making it better suited than pp65 baculovirus-infected cells. When compared to the infection of insect cells with baculovirus, the production of pp65- positive PMNs generated in vitro is more laborious and the transfer protocol needs further optimization. Nevertheless, the use of pp65-positive PMNs generated in vitro is to be the preferred method for production of internal slides.

Whether the sensitivity or the threshold of the laboratories differ cannot be decided. This will be investigated with the distribution of external (patient) standard slides in the near future. This will answer the question whether the assay is to be standardized for comparison of the results.

In conclusion, this study describes the basic requirements towards the standardization of the HCMV-Agemia assay, and the main results are that unfixed standard slides can be distributed at RT provided they are wrapped in aluminium, vacuum sealed, and stored at -80°C upon arrival until staining. The pp65-positive PMNs generated in vitro made by coculture are better suited for standardization purposes than the baculovirus-expressed pp65-positive insect cells. Within individual laboratories, the relative differences between the standard samples were recognized correctly. However, in absolute terms, there were still differences between the participating centres. These differences can be diminished with standard slides as described here so an optimal concordance of test results between different laboratories can be obtained.

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