

Detection of CMV Infected Cells by Flow Cytometry – Evaluation of MAbs CCH2 and AAC10 Directed Against Early and Late CMV Antigens

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Abstract

In this work evaluation of usefulness of monoclonal antibodies (MAbs) CCH2 and AAC10 directed against early – pUL44(DB52) and late – ppUL83(pp65) CMV antigens, utilized in Department of Virology, NIH for routine diagnosis of CMV infection by shell vial and pp65 antigenemia assay, for determination of CMV antigens by flow cytometry in human leucocytes, isolated, infected and cultivated *in vitro* was presented.

Key words: CMV, flow cytometry

Human cytomegalovirus (CMV) still remains a frequent cause of complications in immunosuppressed patients, notably following transplantation and HIV infection. The approaches conducted in aim to control CMV infection has been forward at developing highly sensitive and quantitative methods, allowing to identify infected patients at risk for developing clinical manifestations (Boeckh and Boivin, 1998). Within many different laboratory methods of determination of viral tegument protein – ppUL83(pp65) – commonly named antigenemia assay, is one of the most widespread to confirm active CMV infection (van der Bij *et al.*, 1988). Moreover, antigenemia assay has been successfully introduced for quantifying CMV viral load, however, detection of positive cells by conventional techniques is time-consuming and subjective (Boeckh and Boivin, 1998; Gerna *et al.*, 1990). It seems that these problems can be overcome by flow cytometry and this technique may be ideal method for rapid diagnosis of CMV (Elmendorf *et al.*, 1988; Schols *et al.*, 1989). Despite promising powers, very little studies, especially carried out on clinical materials have been conducted (Imbert-Marcille *et al.*, 1997; Poirier-Toulemonde *et al.*, 2000; Essa *et al.*, 2000).

In this study evaluation of usefulness of monoclonal antibodies (MAbs) utilized in Department of Virology, NIH for routine diagnosis of CMV infection by shell vial and pp65 antigenemia assay for determination of CMV antigens by flow cytometry in human infected leucocytes, isolated and cultivated *in vitro* was conducted.

The experiment was conducted with materials obtained from 4 persons: two CMV seropositive and two seronegative for CMV. Leucocytes were obtained from blood collected on heparine and isolated with procedure according Lymphoprep™ (AXIS-SHELD, Norway) and seeded in 24-well plates at concentration 5×10^5 cells per well in RPMI (Sigma R6504) supplemented with 10% foetal calf serum (FCS, Sigma F2422). Then the cells were infected with CMV AD-169 strain (ATCC, VR-538). The cells were harvested after 24, 48, 72 and 96 hours incubation at 37°C and 5% CO₂, suspended with RPMI + 10% FCS with addition of 5% DMSO, frozen and kept in –70°C before staining.

For staining, washing, permeabilization and fixing, the reagents listed below were used: MAb-Anti-Cytomegalovirus, clone AAC10 (DAKO M7065) reacted with late CMV protein ppUL83(pp65); FITC-Conjugated MAb-anti CMV, clone CCH2 (DAKO F0791) reacted with pUL44(DB52) CMV early protein; FITC-Conjugated F(ab')₂ Fragment of Rabbit Anti-Mouse IgG (DAKO F0313), used for second step staining; γ 2aFITC/ γ 1PE isotype control (BD 340458), negative control for intracellular staining; FACS™ Permeabilizing Solution (BD 340457); Lysing Solution (BD 340182), CellFix (340181 BD); CellWash (BD 349524).

Cells after defrosting (water bath, 37°C) were washed in 2 ml Cell Wash supplemented with 0,5% FCS, centrifugated (5 min, 300 g, room temperature) and permeabilized according producer manual. Then to

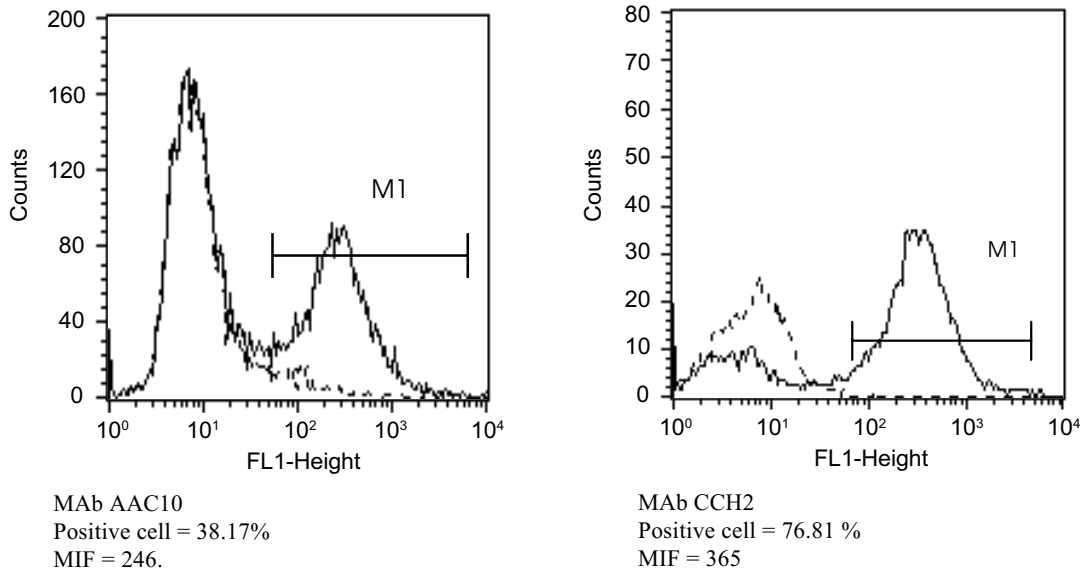


Fig. 1. Fluorescence histograms of CMV-infected MRC5 cells stained with anti-CMV MAbs (—) overlaid with isotype control (-----)

each tube containing approximately 5×10^4 cells the antibodies (AAC10, CCH2 or isotype control) were added. After incubation (30 min) the cells were washed twice and fixed except the tubes contained the leucocytes stained with AAC10 which were stained with second step antibodies conjugated with FITC and then washed twice and fixed. The concentration of antibodies used for intracellular staining was calculated as $1 \mu\text{g}$ IgG for 10^6 cells. In parallel to infected leucocytes several controls were run: non-infected leucocytes obtained, incubated and frozen in the same way as those infected and MRC5 fibroblast mock-infected serving as negative controls and MRC5 infected with CMV AD169 and incubated for 48 h serving as positive control.

Approximately 20,000 MRC5 cells and leucocytes cultivated *in vitro* were analyzed on FACSCalibur flow cytometer (Becton Dickinson). The percentage of positive for CMV cells and the mean fluorescence intensities (MFI) were determined on histograms using a region defined according to isotype control analysis.

Investigated MAbs recognized CMV antigens in infected MRC5 fibroblast. The percentages of positive cells were higher with MAbs detecting early CMV antigen p(UL44) then late pp(UL83) – 76.81% for CCH2 and 38.17% for AAC10, with the higher MIF count for CCH2 also (Fig. 1). These antibodies recognized also *in vitro* infected with CMV human leucocytes. No significant differences (linear regression, $r^2 = 0.9598$, $p < 0.0001$) regarding to detection of CMV positive cells after *in vitro* infection between seropositive and seronegative persons were observed. While the percent of positive cells detected by CCH2 MAb were the highest after 48 h incubation and then were diminished to 36 and 26% after 72 and 96 h incubation, the detection of positive cells by AAC10 MAb was shifted in time at 24 h in comparison to CCH2 and were increased from 34% after 48 h incubation to 52 and 76% after 72 and 96 h respectively (Table I). The mean percentage of cells stained with isotype control and non-infected cells stained with AAC10 and CCH2 MAbs did not exceeded 1,5%.

Table I

The percentage of CMV positive cells obtained from 4 persons after *in vitro* infection and incubation time 24, 48, 72 and 96 h, detected by flow cytometry with MAbs directed to early CMV antigen pUL44 – CCH2 and late CMV antigen ppUL83(pp65)

Patient	CMV serostatus	% CMV positive cells							
		24 h		48 h		72 h		96 h	
		CCH2	AAC10	CCH2	AAC10	CCH2	AAC10	CCH2	AAC10
1	+	2	4	82	42	34	67	35	73
2	+	17	13	90	28	38	45	20	63
3	–	4	9	87	46	33	48	24	91
4	–	6	9	78	20	37	49	23	76

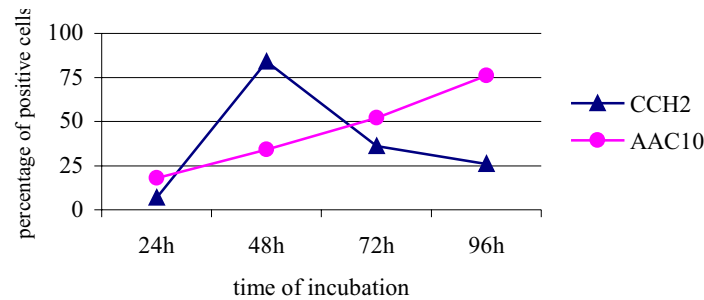


Fig. 2. Time dependent appearance of CMV antigens in human leukocytes isolated, infected and cultivated *in vitro*: early protein pUL44 recognized by CCH2 MAb and late, ppUL83(pp65) recognized by AAC10 MAb investigated by flow cytometry. Percentage of positive cells is expressed as mean value of results obtained from 4 investigated persons

Infected and uninfected cell population can be clearly distinguished by flow cytometry and time dependent appearance of CMV specific antigens can be evaluated as it has been shown by Schols *et al.* (1989). In this experiment this effect can be also observed (Fig. 2). Controversial is higher prevalence of cells harboring late CMV antigen than early one after 24 h incubation. It may be explained by procedural differences in staining (direct for CCH2 and indirect for AAC10) and therefore the higher background or, it may be due to detection of infected cell inoculated to leukocytes.

The most common method used to determine of CMV active infection still remains antigenemia pp65 assay. The procedure of this method is variable and results are dependent on performance of each step: cells isolation and preparation, fixation and staining. The same problems are connected with detection of CMV antigens by flow cytometry and the choice of appropriate MAbs is one of the most important steps. For example, Imbert-Marcille *et al.* (1997) described the 1C3 and C10-C11 pools of MAbs, both directed against pp65 and have the same isotope that indicated differences in labeling. As they concluded, the differences could be explained by recognition of distinct epitopes on pp65 and/or a better penetration of 1C3 antibodies. In this experiment utilization of MAb AAC10 directed against pp65 and MAb directed against early CMV protein DB52, for recognition of CMV antigens in MRC5 cells as well as in human leukocytes by flow cytometry has been confirmed and also no significant differences regarded to detection of CMV positive cells after *in vitro* infection between seropositive and seronegative persons were observed..

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