

J. Kaden<sup>1</sup>, U. Ludwig<sup>2</sup>, R. Preyer<sup>3</sup>

## Serum IgG, IgM, and IgA Antibody Response against Cytomegalovirus-Specific Proteins in Renal Transplant Recipients during Primary and Secondary/Recurrent Infection as Determined by Immunoblotting Technique

A total of 190 selected serum samples from 30 kidney graft recipients obtained in temporal connection with the first occurrence of CMV-pp65-antigen positive leukocytes or IgM-anti-CMV-antibodies were analysed by immunoblot (IB) in a blinded manner. In all sera the number of IgG, IgM and IgA specificities against 8 defined CMV polypeptides and the intensity of reactions were measured. In 12 pretransplant CVM-IgG-EIA(Abbott)-positive recipients antibodies to 150, 78 and 65 kD polypeptides were detectable in 100% of the patients, followed by antibodies to 52 and 38 kD polypeptides in 83% and finally antibodies to 60, 42 and 28 kD polypeptides in 50 – 58% of the patients. The strongest reactions were seen against the 150 kD polypeptide. In 7 out of 18 patients (38.9%) of the pretransplant CMV-IgG-EIA-negative group antibodies to 65 kD polypeptide could be detected by IB. Therefore, the grouping of recipients as CMV antibody positive or negative strongly depends on the test system used. After prophylactic or therapeutic use of human gammaglobulins all recipients became blot positive immediately after infusion. The strongest reaction intensities were found against the 150 kD polypeptide followed by 52, 65 and 38 kD. In 12 recipients suffering from primary CMV infection IgM responses to 65, 52 and 38 kD polypeptides were found in 100%. Most recipients developed also an IgM response to the 150 kD glycoprotein in the course of infection. All recipients with a positive IgM reaction also produced IgA-anti-CMV- antibodies at the same time. The strongest reaction was directed against the 65 kD polypeptide, followed by reactions against 52, 38 and 150 kD polypeptides. With respect to the time point of antigen detection in peripheral blood leukocytes we observed both an increase in number of blot positive recipients and an increase in reaction intensity immediately after occurrence of pp65 positive leukocytes. Remarkable IgM as well as IgA activities were directed against 65, 52 and 38 kD polypeptides. All 12 recipients suffering from CMV reactivation produced IgM responses to 150, 52 and 38 kD polypeptide, most recipients also to 78, 65, 42 and 28 kD polypeptides. All recipients with a positive IgM reaction produced at the same time also IgA-anti-CMV-antibodies. The strongest reaction was directed against the 65 kD polypeptide, followed by reactions to 52, 38 and 150 kD polypeptides. It is worth noticing that in about 70% of recipients suffering from CMV reactivations IgM-antibodies to 150, 52 and 42 kD polypeptides and IgA-antibodies to 52 kD polypeptide could be detected at the time of or shortly before the pp65 test became positive. Therefore, the blotting technique is a valuable and very sensitive tool for detailed characterizing of the CMV antibody status of graft recipients.

**Key words:** kidney transplantation, cytomegalovirus infection, CMV, pp65, immunoblot, Western blot

<sup>1</sup>Vivantes Klinikum im Friedrichshain, Berlin, Germany; <sup>2</sup>Milenia Biotec GmbH, Bad Nauheim, Germany; <sup>3</sup>AID Autoimmun Diagnostika GmbH, Strassberg, Germany

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### **Immunoblot-reaktive IgG-, IgM- und IgA-Antikörper bei Patienten nach Nierentransplantation mit primärer bzw. sekundärer/rekurrenter Cytomegalievirus-Infektion**

*Im zeitlichen Zusammenhang mit dem erstmaligen Nachweis CMV-pp65-Antigen-positiver Leukozyten im peripheren Blut bzw. von IgM-anti-CMV-Antikörpern wurden von 30 Nierentransplantatempfängern insgesamt 190 Serumproben gewonnen und mittels Immunoblot (IB) analysiert. Von allen Seren wurde die Anzahl der CMV-spezifischen IgG-, IgM- und IgA-Banden sowie deren Reaktionsstärke bestimmt. In den Seren von 12 vor Transplantation CMV-IgG-EIA(Abbott)-positiven Patienten waren mittels IB Antikörper gegen die Polypeptide 150, 78 und 65 kD in 100% nachweisbar, gefolgt von Antikörpern gegen die Polypeptide 52 und 38 kD in 83% und gegen die Polypeptide 60, 42 und 28 kD in 50-58%. Die stärkste Reaktion war gegen das 150 kD-Polypeptid gerichtet. Bei 7 von 18 Patienten (38,9%) der präoperativ CMV-IgG-EIA-negativen Gruppe konnten IB-reaktive Antikörper gegen das 65 kD-Polypeptid nachgewiesen werden. Somit hängt die Charakterisierung der Patienten als CMV-Antikörper positiv oder negativ sehr vom verwendeten Testsystem ab. Unmittelbar nach der prophylaktischen oder therapeutischen Anwendung intravenös applizierbarer humaner Gamma-globuline wiesen alle Rezipienten positive Blot-Reaktionen auf. Die stärksten Reaktionen waren gegen das 150 kD-Polypeptid gerichtet, gefolgt von Reaktionen gegen die Polypeptide 52, 65 und 38 kD. Alle 12 Rezipienten mit primärer CMV-Infektion wiesen IgM-Antikörper gegen die Polypeptide 65, 52 und 38 kD auf, die meisten entwickelten auch eine IgM-Antwort gegen das 150 kD-Polypeptid im Verlauf der Infektion. Alle Patienten mit einer positiven IgM-Reaktion produzierten gleichzeitig auch IgA-anti-CMV-Antikörper. Die stärkste Reaktion war wiederum gegen das 65 kD-Polypeptid gerichtet, gefolgt von Reaktionen gegen die Polypeptide 52, 38 und 150 kD. Im zeitlichen Bezug zum erstmaligen Nachweis CMV-pp65-Antigen-positiver Blutleukozyten kam es sowohl zu einem Anstieg der Zahl Blot-positiver Rezipienten als auch zu einem Anstieg der Reaktionsintensität der Banden. Sehr starke IgM- als auch IgA-Antikörperaktivitäten wurden gegen die Polypeptide 65, 52 und 38 kD nachgewiesen. Alle 12 Patienten mit einer CMV-Reaktivierung entwickelten IgM-Antikörper gegen die Polypeptide 150, 52 und 38 kD, die meisten Patienten auch gegen die Polypeptide 78, 65, 42 und 28 kD. Alle Rezipienten mit einer positiven IgM-Antwort produzierten gleichzeitig auch IgA-Antikörper. Die stärkste Reaktion war gegen das 150 kD-Polypeptid gerichtet, gefolgt von Reaktionen gegen die Polypeptide 52, 38 und 150 kD. Bei etwa 70% der Rezipienten mit einer CMV-Reaktivierung ließen sich IgM-Antikörper gegen die Polypeptide 150, 52 und 42 kD und IgA-Antikörper gegen das 52 kD-Polypeptid bereits zum Zeitpunkt des erstmaligen Auftretens pp65-positiver Leukozyten oder sogar kurz vorher nachweisen. Somit ist die Immunoblot-Technik eine wertvolle und sehr empfindliche Methode zur detaillierten Charakterisierung der CMV-Antikörper-Situation von Transplantatempfängern.*

#### **Schlüsselwörter:**

*Nierentransplantation, Zytomegalievirusinfektion, CMV, pp65, Immunoblot, Westernblot*

### **Introduction**

The human cytomegalovirus (HCMV), the most common opportunistic agent post kidney transplantation (post-KTx) and responsible for a high morbidity, induces in the host a variety of antibodies. At the very beginning of investigations the determination of complement-fixing (CF) antibodies was the common used method. These antibodies remain in high titers for many years, indicating chronic or latent CMV infection (1) and were observed also in the absence of viraemia (2). In 1981 we (3) were able to show in 100 cadaveric kidney graft recipients an infection rate of 28% by means of an at least 4-fold increase in CF antibody titer or virus growth in culture (6 out of 28 positive). The disadvantages of the time-consuming and labour-intensive CF antibody determination is the requirement of serum pairs and the impossibility of differentiation between immunoglobulin classes. The introduction of the indirect immunofluorescence technique (IFT) for detection of IgM-anti-CMV antibodies allowed a much earlier serological CMV diagnosis in comparison with the CF-antibody-titer-determination (4). The availability of commercial enzyme-immuno-assays (EIA) was very important with respect to automation, shortened cycle time and an increased throughput, but the determined IgG/IgM-antibodies did not provide information about their functional activities (5). The use of enzyme-linked immunosorbent assays (ELISA) with selected antigens (recombinant polypeptides and glycoproteins) allows the determination of antibodies against "nonneutralizing" and "neutralizing antigens" (6, 7). An alternative method to characterize the antibody variety in a serum is given by the immunoblot technique. Eggers et al. (8) reported on the development of an immunoblot assay for detection of neutralizing antibodies based on the use of recombinant antigens representing neutralizing epitopes of glycoproteins gB and gH of HCMV. In addition, the blot technique is not as time-consuming and labour-intensive as the neutralization assays (9, 10). On the other hand, the blot technique allows elegantly the characterization of the host immune response to different CMV antigens in longitudinal studies (11-17). Müller et al. (18) using a Western blot (WB)

based on recombinant viral envelope (gB and gH) and tegument (pp150 und pp65) proteins could show, that the WB was more sensitive in recognizing IgM-anti-CMV antibodies than ELISA, these IgM antibodies were primarily directed against pp150, but in manifest CMV disease also against gB, and its epitope-specific pattern persisted post-KTx for years. Already in 1992 we (19) reported on different pattern of immunoblot-reactive antibodies in CMV acute phase (p38, p48, followed by p150) and convalescent phase sera (22-26 kD) using an inhouse WB. In the present, more detailed, longitudinal study using at the first time a now commercially available WB we determined serially IgG-, IgM-, and IgA-immunoblot-reactive antibodies against 8 different CMV antigens in 30 kidney graft recipients with or without CMV infection/disease and compared these results with those obtained by ELISA (IgG- and IgM-antibodies) or by IFT (pp65 antigen).

## Material and Methods

### Study population

A total of 30 recipients (mean age, 44.1 ± 11.5 years; females, 9; males, 21) who underwent cadaveric kidney transplantation between March 1994 and November 1997 at the Kidney Transplant Centre Berlin-Friedrichshain were included in this retrospective study. From these recipients the pre- and at least 5 postoperative sera, obtained in temporal connection with the first occurrence of CMV-pp65-antigen or IgM-anti-CMV-antibodies, were analyzed by immunoblot (IB). Thus, a total of 190 selected serum samples, kept at -20 °C in our serum bank, were retrospectively analyzed in a blinded manner.

### Study design

Three groups of recipients were built according to the pre-KTx IgG-anti-CMV-antibody status measured by ELISA.

- A. Donor CMV-IgG-negative [D-], Recipient CMV-IgG-negative [R-]; n<sub>1</sub>=6, n<sub>2</sub>=40 sera.
- B. Donor CMV-IgG-positive [D+], Recipient CMV-IgG-negative [R-]; n<sub>1</sub>=12, n<sub>2</sub>=73 sera.

- C. Donor CMV-IgG-negative [D-], Recipient CMV-IgG-positive [R+]; n<sub>1</sub>=12, n<sub>2</sub>=77 sera.

In all sera the number of IgG, IgM and IgA antibody specificities and the intensity of reactions (semiquantitatively) were retrospectively measured by immunoblot, and in R- recipients also the post-KTx day of their first occurrence was recorded.

This design allows the identification of typical antibody pattern in primary and secondary (recurrent) CMV infections in kidney graft recipients as well as their temporal connection with the first occurrence of IgM-anti-CMV-antibodies measured by EIA or CMV-pp-65-antigen determined by IFT.

In addition, in 15 recipients the influence of intravenously infused human gammaglobulins (CYTOTECT, Biotest, Dreieich, Germany, n= 12; OCTAGAM, Octapharma, Langenfeld, n=3) on the blot-results was checked.

### Immunosuppression

Basic immunosuppression for 29 out of 30 recipients in this study consisted of azathioprine (AZA), corticosteroids and ciclosporine (CY), only one recipient was primarily treated with Mycophenolate mofetil (MMF) instead of AZA. Details of our immunosuppressive regimen have already been published (20, 21). Post-KTx the recipients received 40 mg MP for 7 days, subsequently switching to 35 mg prednisolone for 14 days tapering to 10-15 mg/day. Oral CY was started within 24 hours of surgery. During the first post-KTx week a maintenance CY level of 100 ng/ml and thereafter of 200 ng/ml was given. AZA was started after surgery at a dose of 1 mg/kg per os and maintained as long as the leukocyte count was above 4 Gpt/l.

All recipients have received the Friedrichshain variant of antilymphocyte globulin induction therapy (22) consisting of an intraoperative high-dose single ATG bolus (ATG-Fresenius, Gräffelfing, Germany: n=24; Lymphoglobulin Merieux, Lyon, France: n= 5; RATG Biotest, Dreieich, Germany: n=1) in the operating room before completion of anastomoses (ie, the removal of clamps) via a central venous catheter. To avoid a cytokine release syndrome, 500 mg methylprednisolone

(MP) were given about 60 min pre-ATG.

A total of 8 recipients were switched from AZA to MMF in the postoperative course and 6 recipients were switched from CY to Tacrolimus.

### Rejection

For the diagnosis of rejection, the following clinical and laboratory signs were decisive: enlargement and tenderness of the graft, an increase in serum creatinine, concomitant changes in blood urea nitrogen, oliguria, albuminuria, immunoglobulinuria, sonographic changes and immunoactivation in fine-needle aspiration cytology (23). The treatment consisted of 5 mg/kg bw MP for 5 consecutive days. Biopsy proven MP resistant rejections were tried to reverse by polyclonal antilymphocyte globulin using a dose-by-T-cell protocol (aspired values: 50-150 T-cells/μl). OKT3 (2.5 mg for 10 days; CILAG, Sulzbach, Germany) was given as rescue therapy or primarily in cases of humoral/vascular rejections.

### Cytomegalovirus (CMV) infection

All donors and recipients were screened pre-KTx for CMV-specific IgG antibodies by an EIA. In the D+/R- combination, all recipients received prophylactically CMV immunoglobulins (Cytotect, Biotest, Dreieich, Germany) at postoperative days 1 (2 ml/kg), 18 (2 ml/kg) and 35 (1 ml/kg).

Post-KTx screening was done weekly for IgM and biweekly for IgG during hospital stay. The serological diagnosis of primary CMV infection based on seroconversion and that one of recurrent CMV infection on the detection of CMV-specific IgM antibodies or doubling of CMV-IgG-EIA-Units/ml (IMx CMV IgM and IgG, Abbott, Wiesbaden, Germany).

All recipients were regularly screened post-KTx for CMV-pp65-antigen at least once a week using the commercially available CINAKit (Argene Biosoft, distributed by VIVA, Hürth/Köln, Germany). Details of test performance and results have already been published (24).

The treatment of CMV disease depended on the severity of the clinical symptoms and included the application



Tab. 2: Detailed data of the pre-KTx CMV-IgG-blot positive and CMV-IgG-EIA negative recipients

Molecular weight (kD)	Recipient 1832	Recipient 1864	Recipient 1923	Recipient 1867	Recipient 1879	Recipient 1804	Recipient 1854
	D+R-	D+R-	D+R-	D+R-	D+R-	D-R-	D-R-
	<i>Antibodies of the immunoglobulin classes</i>						
	G A M	G A M	G A M	G A M	G A M	G A M	G A M
150							
78							
65	3 1 -	3 2 -	2 3 2	2 0.5 1	2 - -	1 - 2	1 3 -
60							
52		0.5 - -				1 - 3	- - 3
42		0.5 - -		3 - -			
38		1 - -					
28							

**Legend:** Abbreviations: D+ = CMV-IgG-EIA positive donor, D- = CMV-IgG-EIA negative donor, R+ = CMV-IgG-EIA positive recipients, R- = CMV-IgG-EIA negative recipient, pre-KTx = pre kidney transplantation, G = IgG, A = IgA, M = IgM, the numbers 0.5, 1, 2 and 3 stand for reaction intensities of the IgG/IgA/IgM antibodies (maximum intensity = 4)

Tab. 3: IgA-anti-CMV-reactions (blot) in 5 CMV-IgG-EIA seronegative recipients (combination DR)

Antigen Mol.weight (kD)	Intensity of reaction	pre-KTx	day 9-15 post-KTx	day 16-20 post-KTx	day 21-30 post-KTx	day 31-61 post-KTx	day 66-154 post-KTx
150	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
78	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
65	negative (n = sera)	4	4	4	4	4	4
	weak positive (n)	0	0	0	1	1	1
	strong positive (n)	1	1	1	0	0	0
60	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
52	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
42	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
38	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0
28	negative (n = sera)	5	5	5	5	5	5
	weak positive (n)	0	0	0	0	0	0
	strong positive (n)	0	0	0	0	0	0

**Legend:** The six positive IgA-anti-pp65kD-reactions belong to one patient with no signs of CMV disease (no. 1854). Abbreviation: pre-KTx = pre kidney transplantation

of 19 blot specificities (independent of the immunoglobulin classes) were directed against the 65 kD polypeptide. However, it has to be mentioned that a total of 11 out of 18 recipients of the CMV-IgG-EIA-negative group were also completely antibody negative by immunoblot.

2. *IgG-, IgM- and IgA immunoblot results in 5 recipients of the D-R-group pre- and post-KTx (reproducibility and cross reactions)*

A total of 30 pre- and post-KTx sera from 5 CMV-IgG and IgM-EIA-negative recipients (ie 6 sera per patient) of

kidneys from CMV-IgG and -IgM-EIA-negative donors with no laboratory or clinical signs of post-KTx CMV infection were checked by immunoblot. A detailed analysis of the IgA-blot results can be seen in table 3. The IgG results are not shown, because the post-KTx blot results were strongly influenced by

intravenously infused human gamma-globulins (see next chapter). Table 3 demonstrates the CMV-IgA-blot-results from 5 CMV-IgG/IgM-EIA-negative recipients without any serological or clinical signs of CMV infection. With the exception of one recipient who had IgA antibodies solely to the 65 kD polypeptide (see also table 2, No. 1854) in 6 sera, no IgA antibodies could be determined in the tested sera. This result certifies an excellent reproducibility of the test. This is also proven by the CMV-IgM-blot results of these 5 recipients (not shown as table). Negative results remained negative over the whole period of examination. Interestingly 3 out of 5 recipients (no. 1804, 1854, 1896) showed strong IgM-anti-52 kD reactions and one recipient (no. 1804) an additional weak anti-65 kD reaction already pre-KTx. With respect to cross reactions with Epstein-Barr virus (EBV) it should be underlined that all recipients were routinely screened for EBV antibodies (core IgM, core IgG, early antigen and EBNA) by IFT pre-KTx (26). The detection of IgG antibodies to core antigens and nuclear antigens in all pre-KTx sera of the recipients included in this study proves a past EBV infection. Therefore, as demonstrated by the

CMV blot results, we could not find out cross reactions between EBV and CMV antibodies.

3. *IgG immunoblot results after intravenously infused human gamma-globulins*

Table 4 demonstrates the CMV-blot-specificities and their reaction intensities detected in 12 CMV-IgG-EIA-negative kidney graft recipients who got prophylactically human gamma-globulins (Cytotect, 2ml/kg b.w.) because of CMV-IgG-EIA-positive kidney donors. The number of Cytotect infusions varied between 1 and 3 and the interval between the last Cytotect infusion and the antibody detection varied between 1 and 21 days. In table 4 is shown that almost all recipients became blot positive in that time with respect to the specificities 150, 65, 52, 42 and 38 kD. The strongest reaction intensities were found to the 150 kD polypeptide followed by reactions to 52, 65 and 38 kD polypeptides. In addition, the shorter the interval between infusion and examination the stronger were the reaction intensities. The post-KTx courses of two kidney graft recipients with hypogammaglobu-

linemia and intravenous substitution of normal human gammaglobulins (2 times 10g Octagam) is shown in tables 5 and 6. After substitution in both recipients the CMV-IgG-EIA as well as the immunoblot converted to positive. The blot technique revealed that the sera of both recipients contained antibody specificities to 65 and 52 kD polypeptides, only in one case also antibodies to 42 and 38 kD polypeptides could be detected. All reaction intensities decreased in dependence on the time interval between globulin infusion and antibody testing.

4. *Immunoblot-reactive antibody pattern (IgA, IgM) in recipients with primary (n=12) or secondary/recurrent (n=12) CMV infections in temporal connection to the first occurrence of CMV-IgM-EIA-antibodies*

Seroconversion is the serological evidence of a CMV infection. In order to find out the temporal relationship between blot and EIA results we selected 12 CMV-IgG positive/IgM negative recipients and 12 CMV-IgG-negative/IgM negative recipients and checked at least 5 sera per patient for IgM- and

Tab. 4: Antibody specificities after CYTOTECT-prophylaxis in kidney graft recipients of the D+R- group

Recipient No.	Antibody specificities (according to the molecular weight)								Number of CYTOTECT-infusions prior to antibody detection	Days between last IgG infusion and antibody detection
	150	78	65	60	52	42	38	28		
1832	4	0	3	0	2	1	2	0.5	2	10
1864	4	0	4	0	4	1	4	0	2	5
1923	4	0	3	0	2	0.5	0.5	0	1	18
1867	4	0	4	0	4	4	2	0	2	5
1879	4	0	4	0	4	2	4	0	3	3
1892	3	0	4	0.5	3	2	3	0	2	17
1900	2	0	0	0	0	1	2	0	1	18
1916	3	0	2	0	3	1	2	0	1	18
1933	3	0	2	0.5	2	0.5	2	0	3	21
1934	4	0	2	0	2	1	2	0	2	4
1940	4	0	3	0	3	2	3	0.5	2	1
1953	3	0	0	0	3	0.5	1	0	2	7
positive n=	12	0	10	0	11	9	11	0		
positive %	100	0	83	0	92	75	92	0		
Sum of reaction intensities	42	0	31	1	32	16.5	27.5	1		
Mean reaction intensity	3.5	0	2.6	0.1	2.7	1.4	2.3	0.1		

Legend: The numbers 0, 0.5, 1, 2, 3 and 4 in the columns represent the reaction intensities of the different antibodies

Tab. 5: CMV antibody detection after gammaglobulin substitution (2 x 10g Octagam i.v.) due to hypogammaglobulinemia (5.6g/l)

Recipient No. 1919	Pre KTx	Days post-KTx				
		6	7	13	17	34
CMV-IgG (EIA, AU/l)	0				40	22
CMV-IgM (EIA, OD)	0				0	0
pp65 <sup>+</sup> (IFT, cell count)		0		0		0
Octagam i.v.		10 g	10 g			
CMV-IgG (Blot, MW)						
150 kD	0			2	2	1
78 kD	0			0	0	0
65 kD	0.5			3	3	2
60 kD	0			0	0	0
52 kD	0			3	3	0.5
42 kD	0			0	0	0
38 kD	0			0.5	0.5	0
28 kD	0			0	0	0

**Legend:** Abbreviations: OD = optical density, pre-KTx = pre kidney transplantation, post-KTx = post kidney transplantation. The numbers (0, 0.5, 1, 2, 3) stand for antibody reaction intensities. Positive reactions against the polypeptides 150, 65 and 52 kD six days after IgG infusion.

Tab. 6: CMV antibody detection after gammaglobulin substitution (2x10g Octagam) due to hypogammaglobulinemia (5.3 g/l) and rejection therapy with methylprednisolone (5 x 5mg/kg KG)

Recipient No. 1913	Pre KTx	Days post-KTx					
		15	22	30	31	43	61
CMV-IgG (EIA, U/l)	0	0	0	0			24
CMV-IgM (EIA, OD)	0	0	0	0			0
pp65 <sup>+</sup> (IFT, cell count)		0	0	0		0	0
Octagam i.v.				10 g	10 g		
CMV-IgG (Blot, MW)							
150 kD	0	0	0	0		3	2
78 kD	0	0	0	0		0	0
65 kD	0	0	0	0		3	2
60 kD	0	0	0	0		0	0
52 kD	0	0	0	0		3	2
42 kD	0	0	0	0		2	0.5
38 kD	0	0.5	0.5	0		2	1
28 kD	0	0	0	0		0.5	0

**Legend:** Abbreviations: OD = optical density, pre-KTx = pre kidney transplantation, post-KTx = post kidney transplantation. The numbers (0, 0.5, 1, 2, 3) stand for antibody reaction intensities. Positive reactions against the polypeptides 150, 65, 52, 42 and 38 kD twelve days after IgG infusions.

IgA-blot specificities before and after the first detection of IgM anti-CMV-antibodies by EIA. The antibody pattern is detailed shown in figure 1.

Obviously the antibody detection by blot is possible some days earlier than by EIA.

All recipients (100%) suffering from CMV reactivation produced IgM responses to 150, 52 and 38 kD polypeptides, most recipients also to 78, 65, 42 and 38 kD polypeptides. No reaction was seen to the 60 kD polypeptide.

In recipients suffering from primary CMV infection IgM responses against 65, 52 and 38 kD polypeptides were

found in 100%. Most recipients also developed an IgM response to the 150 kD polypeptide in the course of infection. Only few recipients produced an IgM response to 78, 60 and 42 kD polypeptides, but no reaction was seen against the 28 kD polypeptide.

All recipients with a positive IgM reaction produced at the same time also IgA-anti-CMV-antibodies. Figure 1 demonstrates both an increase in the number of IgA-positive recipients and an increase in the reaction intensities during the course of infection.

The strongest reactions were seen in recipients suffering from reactivations

against the 65 and 52 kD polypeptides, followed by reactions to 150 kD and 38 kD polypeptides, whilst the reactions to 78, 60, 42 and 28 kD polypeptides were only weak.

In recipients suffering from primary CMV infections the strongest reaction is directed against the 65 kD polypeptide, followed by reactions against 52, 38 and 150 kD polypeptides. The reactions against 60 and 42 kD polypeptides were very weak and no reaction was seen against 78 and 28 kD polypeptides.

Detection of IgA and IgM antibodies against 8 different CMV antigens  
by immunoblot in 12 kidney graft recipients with primary and  
12 recipients with secondary CMV infection in temporal relation to the  
IgM-anti-CMV antibody detection by enzyme immunoassay for the first time

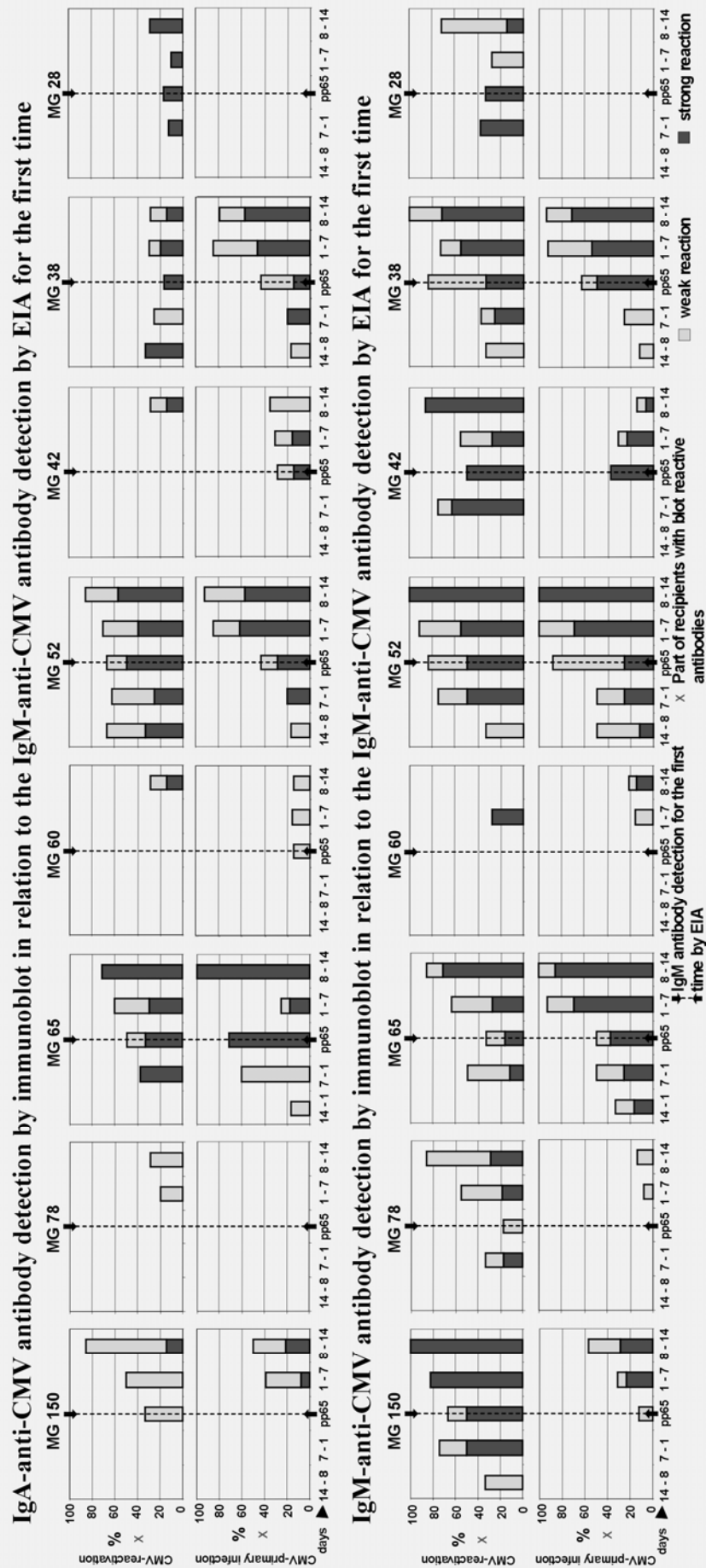


Fig. 1: Immunoblot-reactive antibody pattern (IgA and IgM) in kidney graft recipients suffering from primary (n = 12) or recurrent (n = 12) CMV infections in temporal relation to the first occurrence of IgM-anti-CMV antibodies detected by EIA

Detection of IgA and IgM antibodies against 8 different CMV antigens by immunoblot in 12 kidney graft recipients with primary and 12 recipients with secondary CMV infection in temporal relation to the pp65 antigen detection by immunofluorescence for the first time

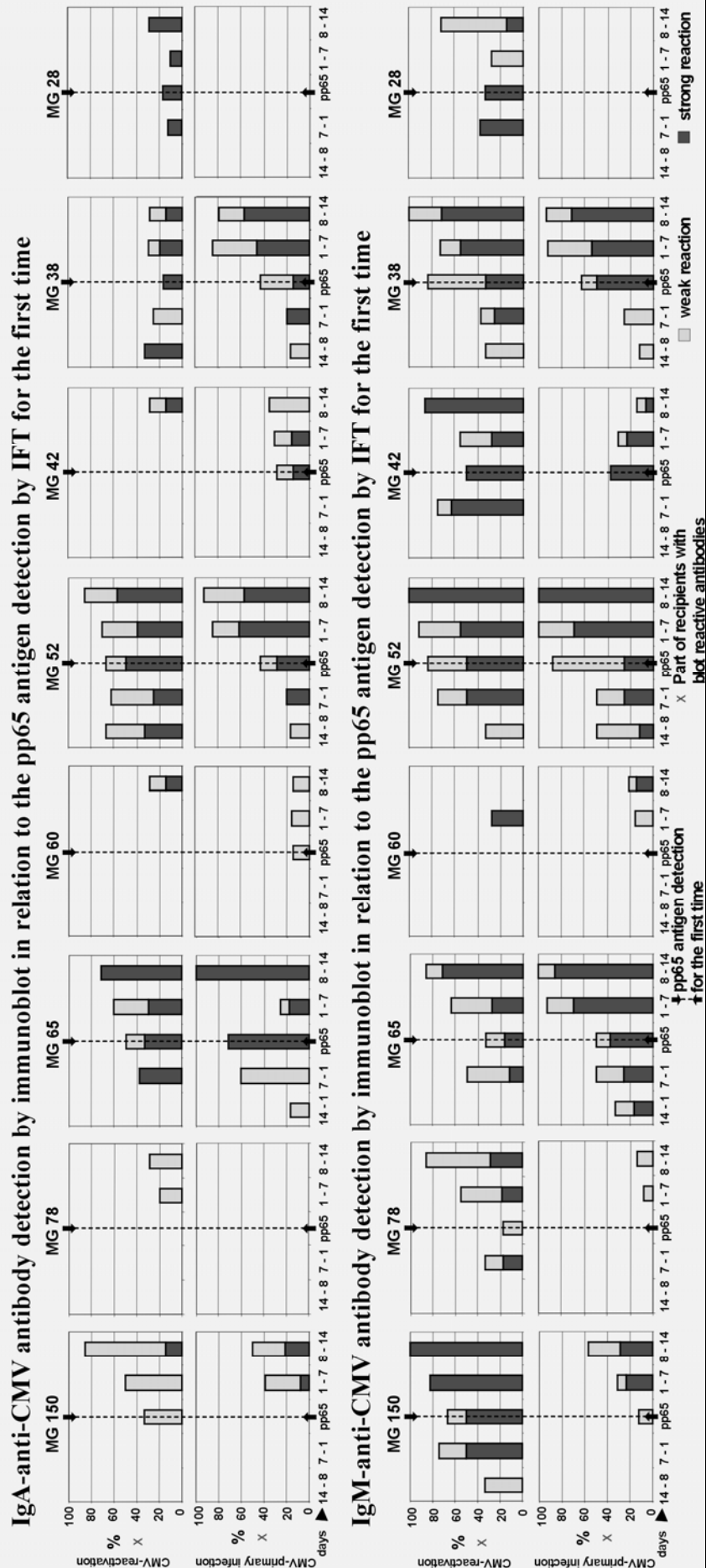


Fig. 2: Immunoblot-reactive antibody pattern (IgA and IgM) in kidney graft recipients suffering from primary (n = 12) or recurrent (n = 12) CMV infections in temporal relation to the first occurrence of CMV-pp65 positive leukocytes in the peripheral blood detected by indirect immunofluorescence

5. Immunoblot-reactive antibody pattern (IgA, IgM) in recipients with primary (n=12) or secondary/recurrent (n=12) CMV infections in temporal connection to the first positive detection of the pp65 antigen by IFT

From the clinical point of view the detection of CMV antigens is the method of choice to diagnose a CMV infection as early as possible. Therefore we compared the IgM- and IgA-blot-specificities against CMV antigens in temporal relationship to the occurrence of pp65 CMV antigen in peripheral blood polymorphonuclear cells. The different pattern of reactions are shown in figure 2.

It is worth noticing that in about 70% of recipients suffering from CMV reactivations IgM-antibodies to 150, 52 and 42 kD polypeptides and IgA-antibodies to 52 kD polypeptide could be detected already some days before the pp65 test became positive. IgM-reactions to 78, 65, 42 and 38 kD polypeptides were seen after the pp65 detection in about 80% of the recipients. 100% of the recipients responded with IgM-antibodies to 150, 52 and 38 kD polypeptides within two weeks after the first occurrence of pp65 antigen. The IgA-reactivation-response in temporal connection with pp65 antigen is rather weak and primarily directed against 65 and 52 kD polypeptides.

In recipients suffering from primary CMV-infections we could observe both an increase in number of positive recipients and an increase in reaction intensities immediately after occurrence of pp65 positive leukocytes. Remarkable IgM as well as IgA activities were directed against 65, 52 and 38 kD polypeptides. No or almost no reactions were seen against 78, 60, 42 and 28 kD polypeptides within the first two weeks after detection of pp65 antigen.

In the fig. 3 and 4 two typical courses of primary and of secondary CMV infection, showing the development of EIA-reactive and blot-reactive antibodies, the occurrence of CMV-pp65 positive blood leukocytes, CMV symptoms and the therapeutic measurements are presented in a synoptic manner. The legends contain some details of both courses.

Serological data in the course of a primary CMV infection post kidney transplantation (patient no. 1916)												
<b>Therapy</b>												
Cytotect (ml)		150			150		140					
Cymevene (mg)							200	400	400	400	400	
<b>Findings</b>												
Temperature (°C)		37,4	36,6	37,6		39,7	38,7	38,2	37,2	37,4	37,7	37,2
Leucocytes (Gpt/l)	5,9		6,4	7,8		3,4	3,8		3,9	3,8	3,5	4,4
ALAT (µmol/l*s)	0,21			0,6		1,29				1,74		0,7
<b>CMV</b>												
IgG-EIA (AU/ml)	0				0		0		67	73	76	98
IgM-EIA (OD)	0				0		0		0	1,3	2,4	3,1
pp65 (cell count)			0	0	0		9		85		12	2
<b>IgG blot</b>												
150 kD	-		3	3	3		3		3	3	3	3
78 kD	-		-	-	-		-		-	-	-	-
65 kD	(1)		3	3	3		2		4	4	4	4
60 kD	-		-	-	-		-		-	-	-	-
52 kD	-		1	1	2		3		3	3	3	3
42 kD	-		2	2	1		1		3	4	4	4
38 kD	-		-	-	2		2		3	4	4	4
28 kD	-		-	-	-		-		-	-	-	-
<b>IgM blot</b>												
150 kD	-		-	-	-		-		(1)	3	3	3
78 kD	-		-	-	-		-		-	-	-	-
65 kD	(1)		(1)	(1)	-		1		4	4	4	4
60 kD	-		-	-	(1)		(1)		(1)	2	3	4
52 kD	-		-	-	2		2		2	3	3	3
42 kD	-		-	-	-		-		-	-	(1)	(1)
38 kD	-		-	-	-		-		-	1	2	2
28 kD	-		-	-	-		-		-	-	-	(1)
<b>IgA blot</b>												
150 kD	-		-	-	-		-		-	1	2	2
78 kD	-		-	-	-		-		-	-	-	-
65 kD	-		-	-	-		2		3	4	4	4
60 kD	-		-	-	-		-		-	(1)	1	1
52 kD	-		-	-	-		-		-	2	2	2
42 kD	-		-	-	-		-		-	(1)	1	(1)
38 kD	-		-	-	-		-		-	1	2	2
28 kD	-		-	-	-		-		-	-	-	(1)
<b>Days postKTx</b>												
	preKTx	3	4	11	18	43	44	45	46	50	53	57
					dis.	reh.						
<b>Intensity of reaction</b>												
	-	(1)	1	2	3	4						

Fig. 3: Development of antibody pattern in the course of a primary CMV infection (no. 1916/97/12)

Basic immunosuppression: Azathioprine (at postoperative day 11 conversion to cellcept), corticosteroids, ciclosporine. Induction therapy with an intraoperative anti-T-lymphocyte-globulin bolus (650 ml, Fresenius, Germany). Immediate graft function. Cytotect infusions as CMV prophylaxis at postoperative days 3 and 18. Discharge (dis.) at postoperative day 19 in a good condition and without any signs of infection, although IgM antibodies to the 52 kD polypeptide were already detectable. Rehospitalization (reh.) 25 days later (postoperative day 43) with fever (39.7 °C), leukopenia (3.4 Gpt/l) and elevated aspartate aminotransferase (0.97 µmol/l x s). By means of indirect immunofluorescence CMV-pp65 antigen positive leukocytes could be seen, but neither IgG- nor IgM-antibodies were detectable by EIA. In contrast, at this time immunoblot-reactive antibodies of all immunoglobulin classes were obviously present (IgM-anti-52 kD and -anti-65 kD antigens, IgA-anti-65 kD antigen). The numbers 1, 2, 3 and 4 in the bars as well as the different grey shade represent the reaction intensities of the different antibodies. After one dose cytotect and a 12 day-cymevene-course the patient was discharged at postoperative day 59 with a serum creatinine level of 118 µmol/(l x s) and no sign of infection. The numbers 1, 2, 3 and 4 in the bars as well as the different grey shade represent the reaction intensities of the different antibodies.

Serological data in the course of a CMV reactivation post kidney transplantation (patient no. 1881)													
<b>Therapy</b>													
Cytotect (ml)						200						180	
Octagam (g)			10										
Cymevene (mg)										150	125	125	125
OKT3 (mg)				2,5	2,5	2,5	2,5						
<b>Findings</b>													
Temperature (°C)							37,6	37,4	37,0	38,3	38,3	39,0	37,5
Leucocytes (Gpt/l)	5,4	8,2	12,1		11,3	10,0	7,1	7,7		4,7		10,9	
ALAT (µmol/l*s)	0,78	0,42				0,52		0,61				0,46	
<b>CMV</b>													
IgG-EIA (AU/ml)	234	185	198			228	>250			>250			>250
IgM-EIA (OD)	0	0	0			0	0,7			1,7			3,1
pp65 (cell count)		0	0		0		6			33			0
<b>IgG blot</b>													
150 kD	4	4	4		4	4		4		4		4	4
78 kD	(1)	(1)	(1)		1	1		2		2		2	2
65 kD	4	4	4		4	4		4		4		4	4
60 kD	-	-	-		(1)	(1)		(1)		(1)		(1)	(1)
52 kD	4	4	4		4	4		4		4		4	4
42 kD	-	-	-		-	-		-		-		-	-
38 kD	-	-	-		4	4		4		4		4	4
28 kD	4	4	4		4	4		4		4		4	4
<b>IgM blot</b>													
150 kD	2	2	2		2	3		4		4		4	4
78 kD	1	(1)	1		4	4		4		4		4	4
65 kD	-	-	-		1	2		2		2		3	2
60 kD	-	-	-		-	-		-		-		-	-
52 kD	-	-	-		2	2		3		3		3	3
42 kD	2	2	2		2	4		4		4		4	4
38 kD	-	-	-		-	(1)		3		3		3	3
28 kD	-	-	-		-	(1)		1		1		2	2
<b>IgA blot</b>													
150 kD	-	-	-		-	-		1		1		2	2
78 kD	-	-	-		-	(1)		1		1		1	1
65 kD	-	-	-		-	-		1		1		3	3
60 kD	-	-	-		-	-		-		-		-	-
52 kD	-	-	-		(1)	(1)		2		2		3	3
42 kD	-	-	-		-	-		(1)		(1)		(1)	(1)
38 kD	-	-	-		-	-		(1)		(1)		1	(1)
28 kD	-	-	-		-	-		-		-		-	-
<b>Days postKTx</b>													
	preKTx	5	12	18	19	23	26	30	32	33	34	37	40
<b>Intensity of reaction</b>													
	-	(1)	1	2	3	4							

Fig. 4: Development of antibody pattern in the course of a recurrent CMV infection (no. 1881/96/39)

Basic immunosuppression: Azathioprine, corticosteroids, ciclosporine. Induction therapy with an intraoperative Lymphoglobulin Merieux bolus (150 mg, France). Acute tubular necrosis. At postoperative day 12 the recipient got 10 g Octagam because of hypogammaglobulinemia of 4.9 g/l. Due to a biopsy proven vascular and cellular rejection an OKT3 therapy was started at postoperative day 18 (treatment protocol: 10 x 2.5 mg). At the same day the recipient received 500 mg methylprednisolone to prevent a cytokine release syndrom and 200 ml cytotect as CMV prophylaxis. The medication of azathioprine was stopped. At the end of OKT3 therapy both CMV-pp65 antigen and CMV-IgM antibodies were detectable (post-transplant day 26). After increase of body temperature and decrease of leukocyte count a CMV therapy consisting of cytotect (one dose of 180 ml) and cymevene (7 days) was started. At the postoperative day 40 the CMV-pp65 test was negative and the patient was discharged at postoperative day 42. Notice the changing blot-reactive antibody pattern in the course of infection, especially the occurrence of IgM-anti-52 kD antigen already at day 19, of IgM-anti-65 kD antigen at day 23, of IgM-anti 38 and 28 kD antigen at day 30 and of five IgA-anti-CMV specificities at day 30. The numbers (1), 1, 2, 3 and 4 in the bars as well as the different grey shade represent the reaction intensities of the different antibodies.

## Discussion

Recipients of organ transplants, subjected to immunosuppressive drugs are particularly susceptible to viral activation or exogenous infection that results in localized or disseminated disease or inapparent infection (27).

Humoral antibodies develop relatively early during infection and persist at high levels during viral excretion. Cellular immunity, however, appears to play the major role in suppression of viral multiplication, leading to latent infection or, less commonly, to viral eradication. Nevertheless, in almost all individuals both CMV-specific immunoglobulins and cellular immunity can be found (28).

The complete virion consists of an inner core (genome, double-stranded DNA molecule), the nucleocapsid (with 162 protein capsomeres) surrounded by the tegument (matrix, about 20 proteins, e.g. pp65 and pp150) and an outer membrane or envelope (lipid bilayer, rev. by 29). Hence envelope proteins (lipoproteins and structural proteins, some of which are glycosylated [glycoproteins]) should be the most relevant antigens for the induction of antibodies. The most immunogenic polypeptides are phosphoproteins of 150, 65, and 28 kD (30). These proteins, however, do not induce neutralizing antibodies. Jahn et al. (31) demonstrated that the 150 kD structural phosphoprotein is outstanding among all viral constituents in eliciting a humoral immune response. In contrast, the antibody reaction against the structural phosphoprotein pp65 (also called lower matrix protein [32]) is rather variable. Meyer et al. (33) pointed out, that four proteins (pp150, pp65, pp58, and pp28) might represent an antigenic complex sensitive enough to detect low levels of antibodies as well as changes in antibody titer due to a past or a present infection. The immunodominant envelope glycoproteins B and H (gB and gH), however, have been shown do induce neutralizing antibodies. Eggers et al. (8) detected anti-gB-antibodies as early as 3-5 months after IgG-seroconversion, indicating that the specific anti-gB-response seems not to be a reliable predictive parameter for fetal infection. On the other hand the determination of glycoprotein-specific antibodies by IB may serve to differentiate between primary and pre-

vious or recurrent infection in early pregnancy. Schoppel et al. (14) using ELISA with selected HCMV antigens observed a delay of 50-100 days in the appearance of glycoprotein-specific antibodies during primary HCMV. Chou (10) using a rapid neutralizing antibody assay found in kidney graft recipients, however, that the pre-existing level of neutralizing antibody did not appear to predict clinical outcome, and moderately high levels of neutralizing antibody did not prevent viremia.

In transplant patients suffering from primary HCMV infection Schoppel et al. (6) reported on rising antibody titers against phosphoproteins pp65 and pp150 at the same time (day 39 post-transplant) as the PCR became positive, whereas glycoprotein titers were not elevated.

In our 12 recipients suffering from primary CMV infection IgM responses to 65, 52 and 38 kD polypeptides were found in 100%. Most recipients developed also an IgM response to the 150 kD polypeptide in the course of infection. Only a few recipients produced an IgM response to 78, 60 and 42 kD polypeptides, but no reaction was seen against the 28 kD polypeptide. All recipients with a positive IgM reaction also produced IgA-anti-CMV- antibodies at the same time. The strongest reaction was directed to the 65 kD polypeptide, followed by reactions to 52, 38 and 150 kD polypeptides. The reactions to 60 and 42 kD polypeptides were very weak and no reaction was seen to 78 and 28 kD polypeptides. With respect to the time point of antigen detection in peripheral blood leukocytes we could observe both, an increase in number of positive recipients and an increase in reaction intensities immediately after occurrence of pp65 positive leukocytes. Remarkable IgM as well as IgA activities were directed to 65, 52 and 38 kD polypeptides.

In 1986 Landini et al. (11) described IgG antibodies reacting with 3 to 5 polypeptides of intermediate molecular weight (82, 74, 66, 62, 55 kD) in addition to pp150 at the time of seroconversion. As p150 and p38 were the two polypeptides constantly and preferentially detected by IgM they suggested to use these antigens in the future as a diagnostic reagent. In 1989 Miller et al. (12) pointed out that in all patients (n=9), the first serum giving a positive results by IB contained IgG to a 66 kD

viral component. In addition, IgM antibodies to 82, 66, and 38 kD polypeptides were very frequently observed. Also Hamann et al. (34) studying 29 renal transplantation patients found that all immunoblot-positive sera responded at least to the 66 kD protein, most of the sera, in addition, also to the 79, 70, 43, 38, 30 or 23 kD proteins. Rautenberg et al. (19) using an inhouse WB identified in acute phase sera early antibodies to protein p38 and p48, followed by high or still rising antibodies to high molecular weight proteins, particularly p150 whereas in convalescent phase sera IgG antibodies directed to 22-26 kD polypeptides transiently appeared. With respect to the immunoglobulin classes Porath et al. (35) described a prominent reactivity of IgM and IgA antibodies to the 66-79 kD and 150 kD polypeptides in the acute sera from all the CMV mononucleosis patients examined, but not in a number of late convalescent sera. Also in renal transplant patients the virus-specific IgM response is primarily directed against the pp150 epitope, but in dependence on the severity of CMV disease the number of specific epitopes involved increase (18). Whereas both IgA1 and IgA2 antibodies with CMV specificity could be detected, IgG1 and IgG3 were the principal anti-CMV subclasses in serum samples from healthy donors and patients (36). With respect to the long-term persistence Eing et al. (37) found during a mean follow-up period of 3.2 years 88% of IgA positive recipients remain IgA positive and 74.8% of IgM positive recipients remain IgM positive and conclude that IgA/IgM-anti-CMV testing for management of CMV infections is dispensable. Also Müller et al. (18) observed the persistence of IgM antibodies against pp150 for years.

The description of variable amounts of secondary bands by the various authors may reflect strain variations, the source of viral proteins, differences in technique, or variations in conditions used for the single technique (rev. by 38).

In post-transplant HCMV reactivation elevated antibody titres were observed at day 29 and included responses against non-neutralizing antigens (pp150, pp65, p52, IE) as well as glycoproteins (14). HCMV positive sera from normal adults prominently precipitated the 64 and the 50 kD polypeptides (13) with additional but fairly consistent pattern of minor bands (69-74

kD, 39-45 kD, 33 and 36 kD). In this connection Zaia et al. (13) pointed out, that bone marrow transplant recipients can make antibodies to individual HCMV proteins in a pattern similar to that displayed by individuals with natural infection. Gold et al. (15) using the IB technique described 3 pattern of antibody responses: 1) early and stable antibody response within one month to 80, 66, 50, 40, 35 and 30 kD antigens in >80% of the sera tested, 2) after 6-8 months antibodies to 100, 60 and 22 kD antigens, 3) antibodies to 115, 63, 58 and 42 kD antigens after 3-4 months with considerable patient-to-patient variability and lower intensity bands.

Van Zanten et al. (17) identified in pre-KTx sera of 11 transplant patients IgG antibodies to pp66 in 100%, and to p150, p104, p94, p50, and p32 in more than 60%. The reactivity pattern of the patients before the transplantation were comparable to those of the healthy individuals. During the course of infection, the reactions against polypeptides already present pre-KTx become more prominent and reactivity against one or more new polypeptides could be found. We serially determined blot-reactive antibodies in 12 recipients suffering from CMV reactivation. All recipients (100%) produced IgM responses to 150, 52 and 38 kD polypeptides, most recipients also to 78, 65, 42 and 28 kD polypeptides. No reaction was seen to the 60 kD polypeptide. All recipients with a positive IgM reaction produced at the same time also IgA-anti-CMV- antibodies. The strongest reaction is directed to the 65 kD polypeptide, followed by reactions to 52, 38 and 150 kD polypeptides. The reactions against 60 and 42 kD polypeptides were very weak and no reaction was seen against 78 and 28 kD polypeptide. It is worth noticing that in about 70% of recipients suffering from CMV reactivations IgM-antibodies to 150, 52 and 42 kD polypeptides and IgA-antibodies to the 52 kD polypeptide could be detected already some days before the pp65 antigen-test became positive.

In our study we also looked for antibody specificities determined by immunoblot and the intensity of reactions in pre-KTx CVM-IgG-EIA-positive and in pre-KTx CMV-IgG-EIA-negative recipients. The main difference between both groups was the occurrence of antibodies to the polypeptides 150, 78, 60 and 28 kD only in the

CMV-IgG-EIA-positive group. The strongest reactions were seen to the 150 kD polypeptide. With respect to the frequency, antibodies to 150, 78 and 65 kD polypeptides were detectable in 100% of patients, followed by antibodies to 52 and 38 kD polypeptides in 83% and finally antibodies to 60, 42 and 28 kD polypeptides in 50-58% of the patients.

In 38.9% of the CMV-IgG-EIA-negative patients blot-reactive antibodies to the 65 kD polypeptide could be detected. Obviously, these antibodies were not able to produce a positive EIA result. The distribution of positive antibody results within the CMV-IgG-EIA-negative sera revealed in 4 out of 7 recipients only one IgG-antibody specificity to the 65 kD polypeptide, but in 3 out of 7 recipients one additional IgG-band (38 or 42 or 52 kD). It should be noticed that all 7 recipients were negative for CMV-IgG and -IgM-EIA-antibodies, pp65 antigen and clinical symptoms. These 7 recipients were categorized as CMV antibody negative by EIA, but the blot results point to contact with CMV. This is also supported by the detection of CMV-IgM-and/or CMV-IgA-antibodies in 6 out of these 7 recipients. Interestingly, in this group a total of 14 out of 19 blot specificities (independent of the immunoglobulin classes) were directed against the 65 kD antigen.

Therefore, the grouping of recipients as CMV antibody positive or negative strongly depends on the test system used (CF, IFT, EIA, blot).

The prophylactic or therapeutic use of human gammaglobulins (especially with a high content of CMV-specific antibodies of the immunoglobulin class G) is very common in graft recipients. Therefore a very careful interpretation of the IgG blot results is necessary. We could impressively show that all or almost all recipients became blot positive immediately after infusion with respect to the specificities 150, 65, 52, 42 and 38 kD. The strongest reaction intensities were found to the 150 kD polypeptide followed by reactions to 52, 65 and 38 kD polypeptides. In addition, the shorter the interval between infusion and examination the stronger the reaction intensities are. All reaction intensities decreased in dependence on the time interval between globulin infusion and antibody testing.

In summary, the immunoblot technique is a valuable and very sensitive tool for detailed characterising of the CMV antibody status of graft recipients as well as for studying the antibody development in the course of CMV infections.

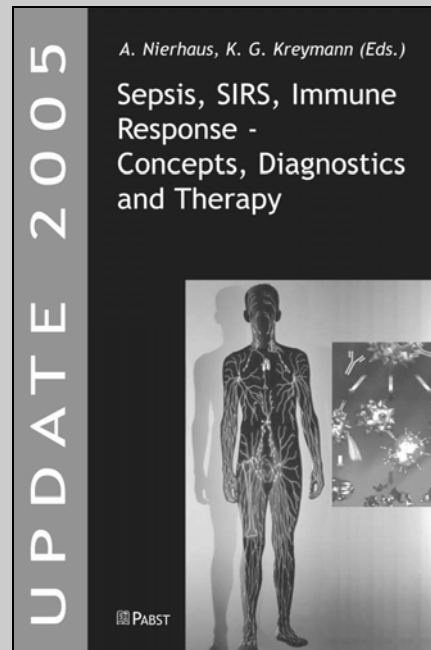
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A. Nierhaus, K. G. Kreymann (Eds.)  
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Concepts, Diagnostics and Therapy**  
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Sepsis and septic shock and the delayed multi-organ dysfunction syndrome (MODS) continue to be the major determinants for adverse outcome in critically ill patients, even though substantial advances in the field of supportive care could be identified recently. However, MODS still remains a serious threat. There is convincing evidence that MODS is essentially the clinical expression of profoundly dysregulated pathways of the innate and adaptive immune system, ultimately leading to organ failure and death.

This book contains the major part of the program of the Fourth International Symposium on "Sepsis, SIRS, Immune Response", held in Hamburg in June 2005.

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Doz. Dr. Jürgen Kaden  
Vivantes Klinikum im Friedrichshain  
Landsberger Allee 49  
D-10249 Berlin  
E-mail: kaden@kaden-dr.de

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