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Detection of virus-specific IgA antibodies in serum of kidney transplant patients with recurrent cytomegalovirus infection by enzymeimmuno and radioimmunoassay techniques

I. SAROV, E. LEVY, M. AYMARD, Y. CHARDONNET, S. BOSSHARD, J.P. REVILLARD, M. FRIEDMAN, E. NORD, M. GREIFF & H. HAIKIN *Departments of Virology, Nephrology and Pathology, Soroka University Hospital and Faculty of Health Sciences. Ben Gurion University of the Negev, Israel, Unite de Virologie, INSERM U.51, CNRS GR 33, Lyon, Clinique de Nephrologie, INSERM U.80, CNRS ERA 792, Lyon, and Bacteriology & Virology Laboratory, University Claude Bernard, Lyon, France*

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SUMMARY

The feasibility of using human cytomegalovirus (CMV) -specific IgA antibody determinations as a signal for early detection of recurrent CMV infections in eight renal transplant recipients was analysed. Solid phase radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase assay (IPA) techniques were used for IgA antibody determinations. In parallel, IgG antibodies to CMV were studied by immunoperoxidase assay. A significant rise of CMV-specific IgG antibody titre was observed in all of these patients between 5 and 53 weeks post-transplantation.CMV-specific IgA antibody production was detected close to the time a rise in CMV IgG antibody was observed in seven out of eight patients studied by RIA and ELISA, and in six out of eight patients studied by IPA. In two patients specific CMV IgA antibodies were detected by all three methods before a significant rise of CMV IgG antibody titre was demonstrated. In these patients CMV IgA was detected by RIA earlier than by ELISA and IPA. The potential application of CMV-specific IgA antibody determination for early detection of recurrent CMV infection in renal transplant patients is discussed.

INTRODUCTION

Cytomegalovirus (CMV) infections affect the majority of renal transplant patients. The incidence has variously been reported from 52–100% (reviewed by Ho, 1977). Depending on the absence or presence of antibody before transplantation, CMV infections have been defined as primary or recurrent. Betts *et al.* (1975) and Ho *et al.* (1975) simultaneously presented evidence that primary infection was associated with the transplant kidney and was independent of the number of transfusions usually administered to transplant recipients. Presumably reactivation of latent virus or in some cases reinfection is the most likely source of recurrent CMV infections after transplantation and immunosuppression. Both primary and recurrent CMV infections can be either asymptomatic or associated with various clinical manifestations including fever, leukopenia,

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Correspondence: Dr I. Sarov, Department of Virology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel.

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pneumonitis, hepatitis, arthralgias, myalgias and shortened graft and patient survival (Betts *et al.*, 1977; Pass *et al.*, 1979).

Laboratory diagnosis of CMV infections in renal transplant patients is usually based on virus isolation or on the complement fixation (CF) test, which has proved to be reliable, though not as sensitive as the indirect immunofluorescent antibody (IFA) techniques (Ho, 1977). These tests, however, only partially fulfill clinical requirements due to the slow growing properties of CMV in cell culture, and the need for appropriately timed paired sera to demonstrate seroconversion or a significant change in the antibody titre.

Demonstration of virus-specific IgM antibodies enables prompt diagnosis with a single serum sample and has been proved to be a valuable diagnostic technique for primary CMV infections (Knez, Stewart & Ziegler, 1976; Schmitz *et al.*, 1977; Gerna & Chambers, 1977; Hekker *et al.*, 1979; Kangro, 1980; Sarov, 1980; Levy & Sarov, 1980; Krishna *et al.*, 1980; Torfason, Kallander & Halonen, 1981).

With regard to recurrent CMV infection the situation is more complex. Kangro (1980), using a radioimmunoassay, found CMV IgM antibody in sera of only one of 13 renal transplant recipients with recurrent CMV infections. Development of serological tests for detection of recurrent human cytomegalovirus infections is of major importance. CMV-specific IgA antibodies have been detected in primary and in some recurrent CMV infections (Schmitz & Haas, 1972: Levy & Sarov, 1980; Torfason *et al.*, 1981; Sarov *et al.*, 1981).

In the present study CMV-specific IgA antibody responses were studied by solid phase radioimmunoassay (RIA), by enzyme-linked immunosorbent assay (ELISA) and by immunoperoxidase assay (IPA) of sera of eight kidney transplant patients who experienced recurrent CMV infection. The results obtained for the sera examined have been compared with specific IgG antibody titres obtained by the indirect immunoperoxidase assay (IPA) (Haikin & Sarov, 1980).

MATERIALS AND METHODS

Antigen preparation. Human embryonic fibroblasts (HEF) were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM L-glutamine, pH 7·2. Before infection, the HEF cultures were split and allowed to grow to confluency. One of the resulting sister cultures was infected and the other was the source of the control antigen. The virus used for infection consisted of a lysate of CMV (strain AD 169) -infected HEF and was used at a dilution of 1:10. The infected and non-infected cultures were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented as above, but with 2% FCS. Both cultures were incubated at 37°C for 9–12 days until advanced cytopathic effects were observed in the infected cultures. The monolayers were washed with 10 ml of cold phosphate-buffered saline (PBS) (pH 7·3), removed by freezing and thawing, sonicated and clarified (1,400 kg, 15 min) as described previously (Friedman, Leventon-Kriss & Sarov, 1979). Protein concentration was determined by the method of Lowry *et al.* (1951). The antigens were devoid of bacterial and mycoplasmal contaminants. CMV infected cells for the immunoperoxidase assay were prepared as previously described (Haikin & Sarov, 1980).

Human sera. This study was performed on 44 sera from eight kidney transplant patients. The patients selected for this study were only those whose sera had been tested for complement-fixing antibody to CMV before or at the time of transplantation and were found seropositive for CMV (titre > 8). Isolation, identification and characterization of human cytomegalovirus from freshly collected urine on cultures of MRC5 human embryo cells was described previously (Revillard *et al.*, 1980; Siqueira-Linhares *et al.*, 1980). All sera were inactivated at 56°C for 30 min, and stored at −20°C until use. Forty-five medical students' sera were used as healthy controls and were treated as described above.

RIA test. The procedure is a modification of that described previously by Friedman *et al.*, (1979). Briefly, 30 μl drops of CMV-HEF or HEF-antigen at the same protein concentration were dried overnight at room temperature in U-shaped polyvinyl microtitre plates (Flow Laboratories, Irvine, Scotland). The following day antigen-coated plates were washed twice with PBS, then filled

with F_{20} PBS (PBS supplemented to 20% with heat-inactivated FCS), and incubated in a humidity box for 1 hr at 37°C. Plates were rinsed, and the diluted sera to be tested were added as described. After 1 hr incubation (37°C), the plates were washed, and 25 μl of ^{125}I-labelled goat anti-human IgA (α chain specific) diluted in FTPBS to $3{\cdot}2 \times 10^5$ c.p.m. per ml was dropped into each well. (The anti-human IgA was obtained from Dakopatts, cat. No. 10.MAT and labelled with ^{125}I by the chloramine-T method of Hunter & Greenwood (1962), at the Nuclear Research Centre, Beer Sheva, Israel, to a specific activity of 20 μCi/μg protein). The serum titre was determined as previously described (Kimmel, Friedman & Sarov, 1980).

Immunoperoxidase technique. The procedure has been previously described (Haikin & Sarov, 1980; Haikin, Leventon-Kriss & Sarov, 1979). Stored slides were thawed, washed in PBS, and covered with test serum or control serum. After incubation at 37°C for 30 min, followed by 15 min in PBS, slides were incubated for an additional 30 min with anti-human IgG peroxidase conjugate (Miles Yeda, Rehovot, Israel), diluted 1:100 in PBS, or anti-human IgA diluted 1:20 (specific for α chain, Dakopatts). After washing, enzymatic activity was detected using a modification of the method of Graham & Karnovsky (1966). The freshly prepared substrate solution was composed of 4 mg benzidine (Riedel de Haen, Seelze-Hannover, FRG) dissolved in 0·5 ml acetone, 9·5 ml PBS, and 10 μl hydrogen peroxide from a 33% stock solution. The substrate was added for 5 min at room temperature (24°C) and was followed by PBS washing. A dark blue stain around the cell membrane and/or nucleus was considered positive. Each test contained known positive and negative sera, and reproducibility of the titration was demonstrated by testing the same positive sera several times.

ELISA. The procedure has been described previously by Levy & Sarov (1980) and Sarov (1980). The assay was carried out on polystyrene, U-shaped microtitre plates of Nunc (microtest 96 U-1182). Amounts of 7·5 μg (0·025 ml) CMV antigen or control antigen per well were allowed to dry overnight at room temperature, and the plates were stored at −70°C. Before use the antigen-coated plates were thoroughly washed with PBST, and this buffer was used for sera and conjugate dilutions as well as for rinsing the plates after each stage of the procedure. Volumes of 0·025 ml human serum dilutions to be tested were dropped into appropriate wells, and the plates were incubated at 37°C for 1 hr. The plates were rinsed and incubated for 1 hr at 37°C with 0·025 ml/well diluted peroxidase-linked rabbit anti-human IgA (specific for α chain) obtained from Dakopatts, Copenhagen. The dilution of the conjugate was 1/50. After 1 hr, the plates were rinsed again, and 0·1 ml of a distilled water solution of 0·08% 5-amino-salicylic acid with 0·005% H_2O_2 (pH 6·1) was added to each well. The enzymatic reaction was stopped by the addition of 0·1 ml NaOH (1 N), and the whole reaction mixture was transferred to tubes and diluted with 1 ml H_2O. Absorbance at 450 nm was measured with a 300 n Gilford Microsample Spectrophotometer. Dilutions of sera beginning with 1:10 were tested on both CMV and control antigen. The serum titre was determined as the intersection between the titration curve using viral antigen and the corresponding curve obtained with the control antigen as previously described (Sarov, Andersen & Andersen, 1980).

In each experiment, known positive and negative sera were included as well as six wells containing only serum diluent. An average background absorption level was determined from these six wells.

RESULTS

Renal transplant patients experiencing recurrent CMV infections

Forty-four sera of eight kidney transplant patients were tested for CMV IgG antibodies by IPA and for CMV IgA antibodies by IPA, ELISA and RIA techniques.

Table 1 demonstrates that a significant rise of specific CMV IgG antibody titre by IPA was evident in all the patients followed after transplantation. In those patients from whom closely spaced serum samples were available, a rise of CMV IgG antibody titre was detectable about 5–12 weeks post-transplantation (patient Nos. 3, 5, 6, 7 and 8). Specific CMV IgG antibody was detectable by IPA against the membrane antigen (Haikin & Sarov, 1980) in all sera tested. In four patients (Nos. 1, 3, 6 and 7) CMV IgG antibodies were detectable against the nuclear antigen(s) as well.

Table 1. Serum CMV antibody titres of kidney transplant recipients who experienced recurrent CMV infections

Patient No.	Age	Time after transplantation (weeks)*	Titres				
			IPA		RIA	ELISA	CMV
			IgG	IgA	IgA	IgA	isolation
1	26	−8	40	<20	<100	<50	
		17	320	4	1600	1600	
		19	320	4	1600	6400	
		24	320	4	1600	1600	
		35	320	4	1600	1600	
		44	320	4	1600	800	
		60	320	4	800	800	
2	28	4	10	<2	<100	<50	
		18	80	4	1600	640	
		30	320	4	1600	640	
		42	80	4	n.d.	640	
		45	80	4	1000	200	
3	27	−1d	10	<2	<100	<50	
		4	20	<2	<100	<50	
		7	160	8	1600	1600	
		9	320	16	1600	400	
		14	160	4	800	100	
		16	80	4	800	100	+
4	52	40	80	<2	400	<50	
		44	80	4	1600	100	+
		48	80	8	n.d.	200	+
		53	320	⩾32	6400	200	
		56	160	16	n.d.	200	
		60	80	8	6400	400	
5	43	2	20	<2	400	<50	
		4	20	4	400	200	
		5	40	4	1600	200	
		12	80	4	1600	800	
		13	80	8	1600	n.d.	
		21	80	16	1600	800	
		52	80	8	1600	800	
6	38	4	10	<2	<100	<50	+
		5	40	<2	<100	<50	
		10	160	8	400	800	+
		14	320	8	1600	800	+
		42	320	4	n.d.	200	
		54	320	4	1600	200	
7	31	−20	40	<2	<100	<50	
		6	160	<2	400	200	
		9	160	<2	400	200	
		24	160	<2	400	200	
8	46	−1d	40	<2	<100	<50	
		6	40	<2	<100	<50	+
		9	320	<2	<100	<50	+

* time given in weeks except when indicated otherwise; d = days; − = before transplantation; + from urine; n.d. = Not done.

In seven of eight renal transplant patients, CMV-specific IgA antibodies in high titre were observed by ELISA and RIA techniques close to the time that a CMV IgG antibody titre rise was seen (patients 1–7, Table 1). Similarly, specific CMV IgA antibody was detected by the immunoperoxidase assay in six out of the eight patients examined (patients 1 through 6, Table 1). A positive reaction was characterized by a partial dark blue ring around the membrane of CMV infected target cells (Fig. 1a). Patient No. 4 produced CMV IgA antibody against the nuclear antigen also (Fig. 1b).

In two patients (Nos. 4 and 5) CMV-specific IgA antibodies were detected by all three methods before a significant rise of specific CMV IgG antibody titre was demonstrated. CMV IgA was detected by RIA earlier than by ELISA and IPA. In one patient (No. 8), no CMV-specific IgA antibodies were detected by any of the three methods. In patient No. 6 a rise of CMV IgG antibody was detected before CMV IgA antibody was detectable.

CMV IgA antibodies were found to persist for a long time. Patient Nos. 1, 4, 5 and 6 were followed for about 60 weeks.

Control healthy adults. Forty-five medical students were tested for their serum titre to CMV. In 32 of them a significant level of antibodies of the IgG class against CMV were found by the indirect immunoperoxidase assay (Haikin & Sarov, 1980), whereas 13 of them were negative. All 45 sera were tested for CMV-specific IgA antibody by IPA and ELISA and proved negative (titres < 2 and < 50 respectively). The possibility that CMV IgA antibodies might be detected at titres lower than 2 and 50 in control sera by IPA and by ELISA respectively needs to be examined. When these control sera were examined for CMV-specific IgA antibodies by RIA, eight out of the 32 positive sera were found to contain a low titre of CMV IgA antibodies (five sera: titre of 100; three sera: titre of 200). The possibility that CMV IgA antibodies may be detected by RIA at titres lower than 100 needs to be examined.

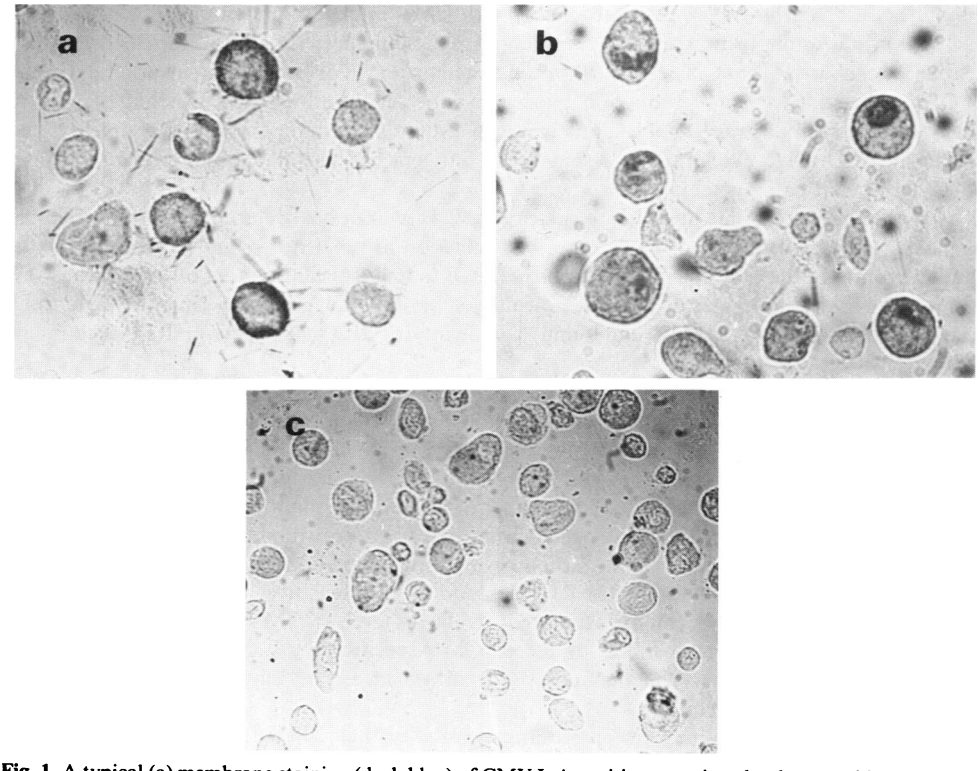

Fig. 1. A typical (a) membrane staining (dark blue) of CMV IgA positive sera given by the peroxidase reaction; (b) nuclear staining of CMV IgA positive sera of patient No. 4, Table 1; and (c) CMV IgA negative sera (titre < 2).

DISCUSSION

The effect of primary and recurrent CMV infection on graft and patient survival after renal transplantation has received increasing attention. In recent studies Whelchel *et al.* (1979) have shown that symptomatic CMV infections occurred in 81% of primary infected and 31% of recurrently infected renal transplant recipients. It has been suggested that the major effect of CMV infection in renal transplant patients is that it suppresses host defences and predisposes the patient to potentially lethal superinfection with other microbial agents (Peterson & Simmons, 1980). This suggestion is consistent with the observation of Hamilton, Overall & Glasgow (1976), who demonstrated a striking increase in mortality due to bacterial and fungal infection in mice undergoing primary infection with murine CMV.

Some of the clinical syndromes which appear following CMV infection are difficult to differentiate from the symptoms of allograft rejection. CMV infection in the presence of augmented doses of immunosuppressive drugs to prevent rejection could transform the disease from a benign to a lethal form. Early detection of CMV infection could be of real value since it would be an indication for a temporary decrease in the immunosuppressive treatment or the use of potentially antiviral drugs which might permit the recipient better to cope with the infection (Peterson & Simmons, 1980; Marker *et al.*, 1980).

Recently, Levy & Sarov (1980), using an ELISA technique have shown that CMV IgA antibodies could be detected in 90% of primary CMV infections. In the present study we have shown that with RIA and ELISA techniques specific CMV IgA antibody production could be detected in the course of recurrent CMV infection in seven out of eight patients examined (Table 1). With IPA, CMV IgA antibody induction was detected in only six patients. With all three techniques CMV IgA antibodies were detectable before a significant rise of CMV IgG antibody could be demonstrated for two patients (Nos. 4 and 5) (Table 1). The earliest detection was obtained by RIA. Thus, CMV IgA antibodies seem not to be restricted to primary CMV infection, but can also be a signal of reactivation and/or reinfection by CMV. A similar situation has been shown by Brunell *et al.* (1975) and by Levy & Sarov (1981), in cases of *Varicella zoster* virus (VZV) reactivation in zoster patients.

Once induced, CMV IgA production appears to continue for a long time, which may indicate that CMV IgA antibody reflects a continued antigenic stimulation in renal allograft recipients. The maintenance of high level of antibody against early antigens has been noted by The *et al.* (1977) in kidney transplant patients they have studied.

It should be noted that in order to adopt CMV IgA antibody determination as a reliable diagnostic tool for detection of recurrent CMV infection in renal transplant patients, a careful standardization of the experimental system is required to find the dilution (cut off) in which CMV IgA antibodies are no longer detectable in healthy subjects. Under our experimental conditions titres of >2, >50 and >200 were found significant for IPA, ELISA and RIA techniques respectively.

In summary the present study demonstrates that specific CMV IgA antibody production occurs in recurrent CMV infection in kidney transplant patients. CMV IgA antibody determination might be useful as a warning signal for detection of CMV infection in these patients. Confirmation of this potential, as an additional serological tool in surveillance programs in renal allograft, awaits examination of serum samples of a larger number of kidney transplant patients.

The IPA, the ELISA and the RIA techniques described are simple and rapid to perform. Antigen for all three techniques can be stored frozen for extended periods, which makes it easily available for diagnostic laboratories. The RIA technique is most sensitive, but the ELISA technique is equally useful, since both techniques detected significant levels of CMV-specific IgA in all eight patients examined, while IPA failed to detect CMV-specific IgA in one patient. For most laboratories the ELISA may be the most convenient since the technique does not require radioisotopes or a gamma counter.

We are currently examining the interesting possibility that CMV IgA antibodies could be detected in recurrent CMV infections in pregnant women. Future studies are also planned to examine the possibility that CMV-specific IgA antibody may be detected in a number of autoimmune or neurological diseases and human cancers in which an aetiological relationship with

CMV has been suggested (Andersen & Andersen, 1978; Kahane *et al.*, 1981; Leonard & Tobin, 1971; Schmitz & Enders, 1977; Dowling, Menonna & Cook, 1977; Link, Wahren & Norrby, 1979; Giraldo, Beth & Huang, 1980; Avni *et al.*, 1981).

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