Cell-Mediated and Humoral Immune Responses to Herpes Simplex Virus and Cytomegalovirus in Renal Transplant Patients

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Cell-mediated immunity to herpes simplex virus and cytomegalovirus, using the lymphocyte transformation test and interferon induction in lymphocytes, was studied in 59 patients from 1 day to 7 years after allotransplantation and compared with the results in normal subjects. Both parameters were permanently depressed with regard to cytomegalovirus. With herpes simplex virus, interferon production was also permanently depressed, whereas the transformation reaction was normal during the first year after transplantation and only slightly depressed in patients more than 1 year after transplantation. In 16 patients the above-mentioned assays and the complement fixation reaction were performed serially and related to the clinical signs of herpes simplex virus and cytomegalovirus infection. The relationship between depression of the transformation reaction and interferon production in lymphocytes and the occurrence of clinically evident herpes simplex virus and cytomegalovirus infections was, however, equivocal. The humoral immune response to herpes simplex virus was measured by the complement fixation test and the more sensitive antibody-dependent, cell-mediated cytotoxicity reaction, and a good correlation was found between these two tests, although only a few persons were found to be negative in the antibody-dependent, cell-mediated cytotoxicity reaction. The suggestion is made that only a few adults are "true" herpes simplex virus seronegative.

Increased frequency and seriousness of infections caused by herpes simplex virus (HSV) and cytomegalovirus (CMV) in renal transplant patients compared to normal persons have been reported by our group as well as by other groups (1, 3, 5, 6, 10, 14, 16).

The humoral immune response in these patients is not suppressed, and the greater susceptibility of transplant recipients to these viruses is generally considered to be due to a defective cell-mediated immune (CMI) response in these patients.

In the present study the lymphocyte transformation reaction and the production of interferon by lymphocytes stimulated with inactivated HSV and CMV antigens were used to measure CMI. The humoral immune response to HSV was studied by the complement fixation (CF) test and the more sensitive antibody-dependent cytotoxicity (ADCC) reaction. The humoral immune response to CMV was examined only by the CF test.

MATERIALS AND METHODS

Patients studied. All patients examined in the study had received a renal allograft at Aarhus Kom-

munehospital, Aarhus, Denmark. The study group consisted of 59 patients, 31 males and 28 females with a mean age of 43 years (16 to 66 years). Of these, 16 (8 males and 8 females) were followed closely from the day of transplantation, using at least three blood samples. The rest of the recently transplanted patients were examined once or twice at various times after transplantation from 1 day to 9 years.

Immunosuppressive therapy. Azathioprine, 2 to 3 mg/kg of body weight, was started on the day of transplantation and continuously given to all patients. Prednisone, 1.5 to 3.0 mg/kg of body weight, was started on day 1 to 3 after transplantation and then slowly reduced to 1 mg/kg after 1 month. Upon suspicion of a rejection crisis, 0.5 to 1 g of methylprednisone was given intravenously daily for 5 to 6 days.

Control group. The control group consisted of 37 persons (14 males and 23 females), laboratory technicians, physicians, and maintenance personnel, with a mean age of 35 years (20 to 56 years).

Blood samples. Fifteen milliliters of heparinized blood (20 IU of heparin per ml) was used for preparation of mononuclear cells, and 5 ml of blood without anticoagulant was used for preparation of serum samples.

Mononuclear cells. Mononuclear cells were obtained by Ficoll-Isopaque flotation and washed as previously described (2). After being washed, the cells were resuspended in RPMI 1640 with buffer and an-

tibiotics and 15% heat-inactivated human HSV- and CMV-negative sera.

Antigen for transformation reaction. (i) **HSV-1.** Human embryonic lung cells grown in Eagle minimal essential medium with 2% calf serum were infected with HSV type 1 (HSV-1) strain MacIntyre (2 to 3 plaque-forming units/cell). At the time of maximum cytopathic effect (24 to 48 h after infection), the culture was frozen and thawed twice and centrifuged at $1,000 \times g$ for 10 min to spin down cellular debris. The supernatant fluid containing 7×10^7 viral plaque-forming units/ml was inactivated by ultraviolet light for 5 min at a distance of 15 cm. After inactivation the virus titer was 0. Control antigen was prepared from uninfected human embryonic lung cells treated in the same way as the infected cells. Antigens were stored in small volumes at -70°C .

(ii) **CMV.** Human embryonic lung cells grown in the above medium were infected with CMV strain Ad 169 (2 plaque-forming units/cell). At the time of maximum cytopathic effect (72 to 96 h after infection) the supernatant fluid was centrifuged at $1,000 \times g$ for 10 min to spin down cellular debris. The supernatant fluid containing 1×10^5 to 1×10^6 viral plaque-forming units/ml was inactivated at 56°C for 1 h. The same control antigen was used for HSV and CMV.

Transformation reaction. After counting, the mononuclear cell suspension was adjusted to contain 10^6 cells per ml and dispensed in 0.2-ml portions in the wells of a microplate (Linbro no. IS-MRC-96). To the cultures was added 30 or 10 μl of undiluted antigen (HSV-1, CMV, or control antigen) and 10 μl of antigen diluted 1:4, 1:16, and 1:64. All determinations were done in triplicate. The plates were closed with plastic covers, and antigen-stimulated cultures and unstimulated cultures were incubated in 5% CO_2 at 37°C for a total of 6 days.

Results of transformation reactions are given as the transformation index (TI), obtained by dividing the disintegrations per minute of viral antigen-stimulated cells by the disintegrations per minute of control antigen-stimulated cells.

Interferon assay. The supernatant fluid from viral antigen-stimulated lymphocytes (10 μl of antigen per 2×10^5 cells in 0.2 ml) was harvested after incubation for 6 days and centrifuged at $3,000 \times g$ for 10 min. The assay was performed by using a micromethod, as previously described (4). The interferon titer was the highest dilution that reduced vesicular stomatitis virus cytopathic effect by 50%.

Characterization of interferons. Interferons were characterized as type I (classical) interferon according to the description given by Valle et al. (18), i.e., partly inactivated after exposure to 56°C for 1 h, no influence by exposure to pH 2 for 24 h, and neutralization with anti-human leukocyte interferon sera.

Target cells for cytotoxicity assays. Skin fibroblasts from healthy human adults, obtained as described by Therkelsen (17), were grown in monolayers in 250-ml Falcon bottles. Two days after being passaged, the cells were infected with HSV-1 strain MacIntyre (1 plaque-forming unit/cell in 5 ml of medium). After 24 h, when about one-third of the cells showed a well-developed cytopathic effect, they were trypsinized, washed, and suspended in Parker medium

TC-199 with 5% heat-inactivated fetal calf serum. Uninfected fibroblasts were treated similarly and used as control cells.

Lymphocytes for cytotoxicity assay. As described in a previous paper on ADCC (9), buffy coat lymphocytes from unknown donors were used as effector cells. Before use the lymphocytes were washed seven times in TC-199 with 5% fetal calf serum. The total amount of lymphocytes obtained from one buffy coat was usually 2×10^6 to 6×10^6 .

ADCC assay. The ADCC assay was performed in conical plastic tubes (Nunc plastic, 11 by 70 mm). To 1 ml of lymphocyte suspension, containing 10^6 cells, was added 60 μl of serum or serum dilution. Target cells labeled with ^{51}Cr by standard methods were added in 0.1-ml volumes from a cell suspension containing 10^5 cells per ml (effector-target ratio, 100:1). Total activity and spontaneous release were determined from tubes containing 1 ml of medium instead of lymphocyte suspension.

The tubes were incubated in humidified air with 5% CO_2 at 37°C for 16 to 18 h. After incubation the tubes were capped, agitated thoroughly, and centrifuged at $200 \times g$ for 10 min; 0.7 ml of supernatant fluid was withdrawn for determination of ^{51}Cr release.

Test tubes with infected target cells were run in duplicate, whereas control tubes were mostly single, because control counts usually were at the same level.

The percentage of ^{51}Cr release from infected target cells minus that from the control target cells gave the percentage of specific ^{51}Cr release (given in figures and table).

CF antibody test. All sera were inactivated and tested for antibodies to HSV and CMV by the CF test. CF antigen was prepared from human embryonic lung cells as described previously (1).

Statistical evaluation. When groups of patients were compared, the Mann-Whitney test was used; the Spearman rank correlation coefficient was used when the results from CF and ADCC examinations were compared.

RESULTS

Cellular immunity to HSV. Lymphocyte transformation in blood samples from various groups of renal transplant patients, compared to blood samples from healthy controls, is given as TI in Fig. 1. A total of 132 blood samples from 50 patients was examined at various times after the transplantation. Results from the first month, 2 to 12 months, and more than 12 months after transplantation are shown. Thirty-seven healthy controls were examined a total of 56 times. Results from both HSV-seropositive and -seronegative (CF test) persons are given. In HSV-seropositive healthy controls the TI was found to be positive (>2) in most blood samples (37 of 38), whereas TIs of blood samples from HSV-seropositive transplant recipients were more often negative (≤ 2). TIs in blood samples from seropositive patients taken more than 1 year after transplantation were found to be sig-

FIG. 1. Lymphocyte transformation of mononuclear cells from blood samples from three groups of renal transplant patients and healthy controls in response to HSV. Symbols: (●) blood samples from seronegative persons; (+) blood samples from seropositive persons; (±) mean $\pm$ standard error of seropositive persons. N, Number of blood samples; Pt, number of patients and healthy controls.

nificantly lower ($P < 0.05$) than those of blood samples from healthy seropositive controls and also lower than in blood samples from patients the first month after transplantation ($P < 0.01$) and 2 to 12 months after transplantation ($P < 0.05$). No differences in TI were found in blood samples from healthy controls and patients the first year after transplantation. Only in a few cases did the TI in blood samples from seronegative individuals exceed 2, and it never exceeded 3.5, irrespective of study group.

The same mean stimulation (disintegrations per minute) in control antigen-stimulated cells was found in the groups of patients and in healthy controls. The mean concentration of viral antigens used to give maximum TI was also found to be the same in the groups of patients and in the normal controls.

The production of interferon in lymphocytes from the same groups of patients and controls after stimulation with HSV antigen is shown in Fig. 2. The interferon produced was in all cases type 1 interferon. Production of interferon was found in lymphocytes from both seropositive and seronegative individuals, and the production in seronegative patients was as high, or higher, than in seropositive patients. When the produc-

tion of interferon in both seropositive and seronegative individuals was taken together, it was found to be significantly lower in the groups of patients examined the first month after transplantation as compared with the patients 2 to 12 months after transplantation ($P < 0.05$) and patients more than 1 year after transplantation ($P < 0.01$). The production of interferon in all three groups of patients was found to be significantly lower than in healthy controls ($P < 0.05$).

Cellular immunity to CMV. Blood samples from the same renal transplant patients and healthy control persons were examined for their cellular immunity to CMV, and results are presented in Fig. 3. In only a few cases did the seronegative individuals reveal a TI exceeding 2, and only in two blood samples from two persons did it exceed 4 (4.7 and 4.3). One of these seroconverted later. In the seropositive individuals the mean TI in all three groups of patients was found to be significantly lower than in the healthy controls ($P < 0.02$). When the TI was compared in the three groups it was found to be significantly lower in patients examined more than 12 months after transplantation (first month and 2 to 12 months compared to more than 1 year: $P < 0.01$). The mean concentration

FIG. 2. Interferon production of mononuclear cells from blood samples from three groups of renal transplant patients and healthy controls in response to HSV. Symbols and abbreviations as in legend to Fig. 1.

of viral antigens used to give maximum TI was found to be the same in the various groups of patients and in the normal controls. It is remarkable that in the first month after transplantation 10 of 28 patients were found to be seronegative, whereas only 2 of 27 patients were found to be seronegative in the group of patients examined more than 1 year after transplantation. In the healthy controls half of the persons were found to be seronegative.

Production of interferon in lymphocytes stimulated with CMV antigen was only rarely found in seronegative patients (Fig. 4). Interferon production was found more often in blood samples from seropositive than in those from seronegative healthy donors, and at a significantly lower mean titer ($P < 0.01$) in blood samples from seronegative donors. During the first month after transplantation, interferon was found in only one blood sample from a seropositive patient. Later, the mean titer of interferon was always found to be significantly lower in patients than was the mean titer in healthy controls ($P < 0.001$).

Humoral immunity to HSV. Complement-fixing and ADCC-reactive antibodies to HSV in sera from renal transplant patients and from

healthy controls were compared. Results are shown in Fig. 5.

A good correlation was found between CF antibodies and ADCC antibodies ($P < 0.001$), although 25 of 40 CF-negative sera were found to have an ADCC activity higher than 10%; thus, low ADCC activity in sera from CF-seronegative individuals was only found in a few persons from each group. A slightly higher activity in the ADCC test was found in sera from normal persons than in sera from patients (not significant).

Humoral and CMI responses and clinical symptoms. Among the 16 patients studied longitudinally after transplantation, 3 patients showed a fourfold rise in CF titer to HSV. One of these patients had eruption of cold sores, and another had a prolonged HSV ulcer on the lower lip; the third patient did not show any apparent signs of HSV infection. All three patients displayed an increase in TI and interferon production, especially the patient who had a prolonged HSV ulcer.

Seroconversion to CMV or a fourfold rise in CF titer was seen in 11 patients. The results from the longitudinal study of CF antibodies, interferon, and TI are shown in Table 1. In all the patients with seroconversion (patients 1-5)

FIG. 3. Lymphocyte transformation of mononuclear cells from blood samples from three groups of renal transplant patients and healthy controls in response to CMV. Symbols and abbreviations as in legend to Fig. 1.

clinical signs of CMV infection were seen. In only one of the originally seropositive individuals (patient 6) where a fourfold rise in CMV titer was seen were symptoms noted which could have been due to a CMV infection. Evidence of cellular immunity began to appear in connection with resolution of illness in five of six individuals. In four of five patients who seroconverted against CMV, production of lymphocyte interferon by stimulation with CMV antigen was found after seroconversion. Unfortunately, one patient (no. 1) was not tested after his seroconversion. In only two of five patients showing a significant increase in titer, but not having signs of CMV infections, was a CMI reaction seen in the first week after transplantation.

DISCUSSION

Clinical manifestations of herpesvirus infections in immunosuppressed patients have been dealt with in many publications (1, 3, 5, 10, 11, 13).

Andersen and Spencer (1) reported in 1969 several cases of CMV infections in renal transplant patients. In the same group of patients a

greater frequency of varicella-zoster infections was seen, which contrasted with a normal incidence and a rather normal clinical course of HSV infections (16). In a later publication, Korsager et al. (6) reported on prolonged and widespread infections with HSV in renal transplant patients. This was seen during a period when rejection crises were treated with massive doses of prednisone or prednisolone or both. In recent years, after reduction in the dosage of these drugs, the occurrence and clinical course of herpetic eruptions have been similar to previous experience (Spencer, unpublished data). In cardiac transplant patients, Merigan's group (13) found a higher incidence, together with a prolonged and more widespread clinical course, of herpesvirus infections than in control persons and also observed several cases of CMV infections (11).

The humoral immune response to these infections in transplant patients has always been found to be as high (13) or higher than that found in normal persons (1, 8, 11, 12). In our patient group we used the sensitive ADCC reaction for HSV and found slightly higher reactivity in healthy controls than in renal transplant

FIG. 4. Lymphocyte transformation of mononuclear cells from blood samples from three groups of renal transplant patients and healthy controls in response to CMV. Symbols and abbreviations as in the legend to Fig. 1.

FIG. 5. Relation of CF and ADCC-reactive antibodies to HSV in sera from renal transplant patients (+) and healthy controls (O). CF antibody is given as the reciprocal of the serum dilution, and ADCC is given as percentage of cytotoxicity in a ^{51}Cr release.

TABLE 1. Humoral and cellular immunity in 11 patients with a fourfold rise in CF titer or seroconversion to CMV

Patient	Time after transplantation														
	0-1 wk			2-4 wk			5-8 wk			9-16 wk			17-24 wk		
	CF	IF ^a	TI	CF	IF	TI	CF	IF	TI	CF	IF	TI	CF	IF	TI
1. L.L.J. ^b	<4	<3	<2	<4	<3	<2	32	<3	<2	ND ^c	ND	ND	ND	ND	ND
2. L.B.H. ^b	<4	<3	<2	<4	<3	<2	32	<3	<2	32	<3	<2	16	720	24
3. B.H. ^b	<4	ND	ND	<4	<3	<2	<4	<3	<2	64	240	<2	— ^d	ND	ND
4. G.S. ^b	<4	ND	ND	<4	<3	<2	8	<3	<2	64	<3	5.2	256	27	2.5
5. E.J. ^b	<4	ND	ND	32	<3	<2	≥256	<3	33.7	≥256	9	5.0	ND	ND	ND
6. A.L. ^b	32	<3	2.5	16	ND	ND	16	ND	ND	≥256	81	<2	64	81	3.2
7. J.C.	16	ND	ND	16	<3	<2	32	<3	<2	128	27	<2	128	720	<2
8. M.N.	32	<3	<2	16	<3	<2	≥256	<3	3.3	≥256	<3	<2	128	<3	<2
9. J.S.	64	<3	<2	64	<3	<2	256	<3	<2	≥256	720	2.1	ND	ND	ND
10. L.G.	16	<3	19.1	16	<3	3.4	ND	ND	ND	64	<3	2.8	128	<3	2
11. J.S.P.	128	240	4.6	128	<3	<2	128	<3	3.2	256	<3	<2	ND	ND	ND

^a IF, Interferon.^b Signs of clinical CMV infection, primarily fever, seen in patient 1, 5 to 7 weeks post-transplantation (PT); in 2, 3 to 4 weeks PT; in 3, 6 to 9 weeks PT; in 4, 4 to 8 weeks PT; in 5, 8 to 12 weeks PT; and in 6, 6 to 9 weeks PT.^c ND, Not done.^d —, Patient died. Cause of death was a stop in graft function developed after clinical CMV infection.

patients.

The good correlation between CF and ADCC antibody levels may indicate that the same antibodies take part in both reactions. It is interesting that only a few healthy controls and graft recipients had cytotoxicity values around zero. This might indicate that only a few humans are "true" HSV seronegative. Some CF-seronegative individuals might have had an infection in early childhood. The cytotoxic reaction in CF-seronegative persons might, of course, also be due to cross-reactivity to other herpes-group viruses in the ADCC reaction, but unfortunately it has not thus far been possible to perform this reaction with CMV and varicella-zoster virus.

No differences in the HSV TI were seen in patients during the first year after transplantation and in normal controls, whereas a significantly lower TI was found with HSV more than 1 year after transplantation and in all patient groups when CMV was used as antigen. The lowest TI was seen more than 1 year after transplantation with both HSV and CMV antigens. This could be due to reinfections or new infections with CMV and perhaps also to activation of HSV and CMV infections, especially during the first months after transplantation, resulting in CMI stimulation. This explanation is supported by the finding that the number of CMV-seronegative patients steadily decreased during the months after transplantation, an observation which has been reported previously (1, 7). It is also supported by data from the 16 patients who were followed longitudinally. Five of these seroconverted and six had significant rises in CF

antibodies during the first few months after transplantation, and some had a rise in the TI.

A depression of TI for CMV has been found in previous studies of heart transplant patients (11) and renal transplant patients (7, 15). In heart transplant patients Merigan's group (13) also found a depression of TI for HSV early after transplantation, which later became normal. Differences in immunosuppressive therapy, especially the use of antilymphocyte serum in heart transplant patients during the first 3 months after transplantation, could explain these differences.

The interferon produced in lymphocytes on stimulation with HSV and CMV antigen was nonimmune or classical interferon in all cases. Its role in the cellular immune response is still a matter of debate. On stimulation with HSV, production of interferon was found in both seropositive and seronegative healthy persons and patients. Nearly the same titer was found in the seropositive and seronegative persons. These results are rather difficult to explain if production of interferon is an immunological parameter, but they might be explained if only a few persons are "true" seronegatives. On stimulation with CMV, production of interferon was found on only one occasion in a seronegative patient, and the titer was significantly lower in seronegative than in seropositive healthy donors. Although we perhaps were using a less potent antigen in our CMV studies than in our HSV studies, these findings may indicate that production of interferon in one way or another is connected to immunity. The production of interferon in lym-

phocytes in patients after seroconversion also confirms this.

With both antigens a significantly lower production of interferon was found in graft recipients as compared with healthy controls, and the most severe depression was seen during the first month after transplantation. These findings are in agreement with those of Merigan's group, although they found normal interferon production with HSV in patients more than 6 months after transplantation (13). They had many cases of mucocutaneous HSV infections the first month after transplantation and suggest that this may be the basis for a normal reactivity to this antigen with increasing time after transplantation.

Another possible explanation for the decline in TI and the rise in interferon production after transplantation for both viruses could be differential acute and cumulative effects of the immunosuppression therapy upon T cells (TI) versus B cells (interferon production).

In the small group of patients followed more closely after transplantation, clinical symptoms of CMV infection were seen particularly in those patients seroconverting, whereas symptoms of HSV infections were found only in the seropositive group. The clinical course of the HSV infections was not different from that in normal persons. The CMI to specific antigens in the seropositive patients before symptoms of CMV and HSV appeared was, judged from our parameters, not different from the CMI in patients without clinical symptoms of disease, but the number of our patients was too small to draw conclusions.

The connection between the depression in TI and interferon production in lymphocytes and the occurrence of clinically evident HSV and CMV infections cannot be analyzed further from the present results. Previous workers have tended to find this connection rather close (7, 11, 13, 15). We think it is important to study still more patient groups with CMI defects and more "normals" with clinical diseases, and also to use more parameters of CMI. Lymphocyte-mediated cytotoxicity reactions are possibly especially important to examine, and particularly it would be of interest to examine the action of lymphocytes from different patient groups and normal individuals in the ADCC reaction.

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