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CELL-MEDIATED AND HUMORAL IMMUNE RESPONSES OF RENAL TRANSPLANT RECIPIENTS WITH CYTOMEGALOVIRUS INFECTIONS

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SUMMARY

Cell-mediated and humoral immune responses were determined in immunosuppressed renal transplant recipients. A micro-phytohaemagglutinin (PHA) stimulation test utilizing whole heparinized blood and a macro-PHA test utilizing separated, washed lymphocytes were used to study cell-mediated immunity. Humoral immune status was estimated by determining quantitative immunoglobulins and complement fixing (CF) antibody titres to cytomegalovirus (CMV) infection. Micro-PHA responses were found to be markedly depressed in patients undergoing immunosuppressive therapy and in patients with chronic uraemia. Macro-PHA responses were normal, indicating that serum factors were responsible for the depressed micro-PHA responses. Antibody responses to CMV infections were found to be ten to a hundred times higher than in normal persons. An inverse relationship was demonstrated between micro-PHA responses and peak CF antibody titres to CMV infections. Humoral immune responses appeared to compensate for depressed cell-mediated immunity as measured by the micro-PHA test. Four patients had very low micro-PHA responses, did not respond to their CMV infections with CF antibody, and died of mixed bacterial and viral infections. Serum immunoglobulins of two were studied and were shown to be greater than two standard deviations below the normal mean. These patients appeared more suppressed than other patients receiving similar therapy and thus probably retained higher concentrations of suppressive drugs.

INTRODUCTION

Current programs for renal allograft transplantation rely to a large degree on immunosuppressive therapy to circumvent rejection. At the dosages routinely used, the immunosuppressive regimens, particularly those including antilymphoblast globulin (ALG), have been shown to depress markedly the expression of cell-mediated immunity (Levin, Landy &

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Frei, 1964; Monaco, Wood & Russell, 1967; Volimer, 1951; Waksman, Arbouys & Arnason, 1961), while leaving humoral immunity relatively, though not totally, intact (Brent, Brown & Medawar, 1958; Merrill *et al.*, 1961). While depression of cell-mediated immunity is necessary to circumvent rejection of incompatible grafts, the severe and generalized deficits caused by these drugs have also been associated with an increase in clinical infection with a variety of pathogens including bacteria, fungi and viruses (Bach *et al.*, 1973; Craighead, Hanshaw & Carpenter, 1967, Fine *et al.*, 1972; Rifkind, Goodman & Hill, 1967). We have recently reported (Lopez *et al.*, 1972) that herpesviruses, especially cytomegalovirus, are associated with severe infections in recipients of renal allografts undergoing immunosuppressive therapy which included ALG. Since the immunosuppressive regimen used to treat these patients was tailored to the degree of antigenic incompatibility and was administered on the basis of body weight or body surface area, it seemed relevant to analyse the effect of immunosuppression on both humoral and cell-mediated immunity and to further correlate the relationship of serious virus infections to the degree of immunosuppression of humoral and cell-mediated immunity in these patients.

MATERIALS AND METHODS

The study group consisted of sixty-one patients who received renal allografts at the University of Minnesota hospitals. Forty-eight of these patients were admitted to this study when re-hospitalized for fevers or rejection episodes while the remaining thirteen were randomly selected for serial study prior to transplantation. The procedures for the selection of donors and recipients, techniques of transplantation, standard immunosuppressive regimens and diagnosis and treatment of rejection episodes have previously been described (Simmons, Kjellstrand & Najarian, 1972). Briefly, the immunosuppressive regimen for recipients of cadaver kidneys included ALG for 14 days, large doses of methylprednisolone on the day of and 2 days following transplantation, high doses of azathioprine and prednisone for several days, and then decreasing amounts to maintenance doses. Repicients of living related donor kidneys, in addition to the above therapy, received ALG for 2 days before as well as 14 days after surgery.

The virus infections in this study group have been described elsewhere (Lopez *et al.*, 1974). Laboratory evidence of virus infection included isolation of virus from patients' secretions and identification by standard methods and/or demonstration of a four-fold or greater rise in serum complement fixing (CF) antibody titre. Forty-seven of the sixty-one study group patients demonstrated laboratory evidence of cytomegalovirus (CMV) infections, fifteen of herpes simplex virus (HSV) infection, seven of herpes zoster virus (HZV) infection, and eight had no evidence of virus infection.

Serum and heparinized blood were collected at weekly intervals during the 2–4 weeks after the patient was started on the study and then once every 4–8 weeks thereafter. Cell-mediated immunity was assayed by *in vitro* lymphocytic responses to phytohaemagglutinin (PHA) using the micro-PHA test described by Park & Good (1972) and a commonly used macro-PHA test (Hadden *et al.*, 1972). The micro-PHA test utilizes heparinized whole blood and gives a quantitative estimate of the patient's thymus-dependent cell (T-cell) function in peripheral blood. Since the test is conducted in the patient's own serum, lymphocytic responses, as well as serum factors which may affect their responses, contribute to the final result. The macro-PHA test was carried out with slight modifications of a previously

described method (Hadden *et al.*, 1972). Modifications included using $0{\cdot}5 \times 10^6$ cells per 0·5 ml, incubation of cells in Dulbecco's modified Eagle Medium with 10% foetal calf serum and antibiotics, and measuring acid insoluble radioactivity in 10% Bio-Solv in Ready-Solv (Beckman Instruments, Palo Alto, California) in a Beckman Model LS-250 liquid scintillation counter.

Humoral immune status was estimated by determination of quantitative immunoglobulins and complement fixing (CF) antibody titres in response to virus infections. CF antibody titres were determined to CMV (strain AD-169), HSV and HZV (Lennette, 1969)). Radial immunodiffusion was used to determine serum concentrations of IgG, IgM and IgA (Mancini, Carbanara & Heremans, 1965). Normal means $\pm$ standard deviations were 1158 ± 305 mg per 100 ml for IgG, 99 ± 27 mg per 100 ml for IgM, and 200 ± 64 mg per 100 ml for IgA.

RESULTS

More than 500 micro-PHA tests were carried out on the sixty-one study group patients. Although great variations were found in PHA stimulation responses of individual patients, almost all responses were lower than those found in healthy controls. Normal mean response in the PHA stimulation test is $7650 \pm$ SD 2615 cpm (Park & Good, 1972). When micro-PHA stimulation responses of the sixty-one immunosuppressed patients were determined while the patients were stabilized on maintenance doses of azathioprine and prednisone, the mean response was $1436 \pm$ SD 1708 cpm. The mean response is greater than two standard errors below the mean of the normal ($P < 0{\cdot}0001$). The patients who had recovered from viral infections had a mean response of 1556 cpm which is not signficantly different from the entire patient group or a group of eight patients who never developed virus infections (1547 cpm). In addition, significant changes in the micro-PHA responses, attributable to the CMV infections, were not detected during the course of those infections.

Although viral infections did not affect micro-PHA responses, the latter were affected by uraemia and the immunosuppressive drugs administered. Five patients were studied 3–4 weeks prior to their engraftment to determine the effect of uraemia on the micro-PHA response. The average micro-PHA response of this group was 244 cpm (Fig. 1a) which is significantly lower than the average response of immunosuppressed patients. When these patients were dialysed repeatedly to prepare them for surgery, the average micro-PHA response rose to 5518 cpm (a normal response) (Fig. 1b). Micro-PHA responses dropped again when the patients were placed on immunosuppressive drugs (249 cpm, Fig. 1c) and rose when the doses of immunosuppressive drugs were gradually reduced (Fig. 1d). By way of comparison, the micro-PHA responses of nephrectomized donors fell from a mean of 5500 cpm to 1540 cpm on the 1st day after surgery, but returned to normal within 2 days, indicating a transient immunosuppression caused by the surgery. In addition, patients undergoing intensive immunosuppressive therapy for rejection episodes always showed marked decreases in their micro-PHA responses detected within 1 day after therapy was initiated. Conversely, when the immunosuppressive therapy was stopped in five patients who rejected their transplanted kidneys, micro-PHA responses of four of these patients returned to normal ($\pm$ 1 standard deviation of normal mean) within 1 week, and in the other within 4 weeks after therapy was stopped.

A different pattern was seen in the four patients who died in the first 3 months after surgery.

Each died of mixed bacterial and viral infections and had extremely low responses in the micro-PHA stimulation test (usually <400 cpm) throughout their course. They showed no increase in PHA responses when immunosuppression was reduced or even stopped. In addition, their kidneys showed no evidence of renal allograft rejection at postmortem examination despite long periods off the immunosuppression prior to death. Low micro-PHA response, however, could not always be correlated with increased susceptibility to severe infection or failure to reject renal allografts. Thus, it was not unusual for patients whose clinical courses were free of serious clinical infection to have periods of weeks with equally low responses. In this latter group, however, micro-PHA responses eventually rose

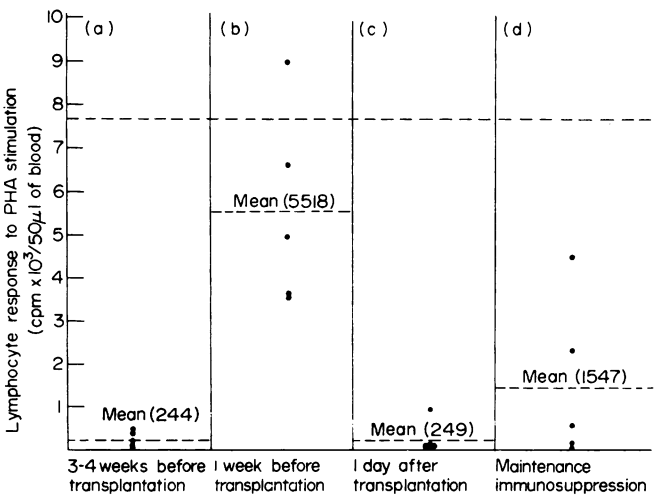

Fig. 1. Micro-phytohaemagglutinin stimulation test responses of five patients (a) 3–4 weeks prior to transplantation and thus with chronic uraemia, (b) 1 week before transplantation and after extensive dialysis in preparation for surgery, (c) 1 day after transplantation and thus after administrations of immunosuppressive drugs, and (d) on maintenance doses of immunosuppressive drugs. (- - -) Mean of normal value ± standard deviation is 7650 ± 2615 cpm.

to levels equalling or exceeding mean responses for patients on maintenance doses of immunosuppressive therapy. Also, rejection episodes occurred in these patients despite moderately low micro-PHA responses. Predicting susceptibility to infection or to rejection by a single or even a number of sequential PHA responses was not possible. The only correlation that was found was that patients who died early in their course never demonstrated PHA responsiveness of any magnitude and the patients who did not die ultimately demonstrated some PHA responsiveness, however modest.

The macro-PHA test was carried out on purified and repeatedly washed lymphocytes of heparinized blood samples collected from nine patients. Normal responses in this test is 48,456 cpm. Eight of the samples were collected during the first 2 weeks after transplantation and while the patients were receiving ALG, and five were collected while the patients were on maintenance doses of prednisone and azathioprine. The average response of the immunosuppressed renal transplant patients on maintenance therapy was 50,283 cpm, while the average response during the first 2 weeks after transplantation was 46,895 cpm. These

responses can therefore be considered normal. One of the four patients who died of mixed bacterial and viral infections and who demonstrated a micro-PHA response of only 95 cpm 4 days before death, had a macro-PHA response of 45,478 cpm on that same day.

Serum immunoglobulins were quantitated in at least two samples from each of seven patients with laboratory evidence of CMV infections (Table 1). In two of these patients the CMV infection was associated with pneumonia and death due to mixed bacterial and viral infections. In addition, one patient had a protracted course due to CMV-associated hepatitis, and the other four patients had only mild febrile or subclinical illnesses associated with their CMV infections. Laboratory evidence of the CMV infections included repeated isolation of the virus from urine and sputum cultures in each patient and, in four of these patients a four-fold or greater rise in serum CF antibody titres. Virus was repeatedly isolated from two patients who never produced detectable CF antibody to CMV and from one patient who did so 1 year after virus was first isolated.

TABLE 1. Serum immunoglobulins and complement fixing antibody titres during virus infections in immunosuppressed renal transplant patients

Patient	Serum immunoglobulins* (mg/100 ml)			Cytomegalovirus complement fixing antibody titres
	IgG	IgM	IgA	
R.S.	2240	450	370	$\geq$1 : 4096
E.B.	1740	165	355	1 : 256
K.L.	1740	115	125	$\geq$1 : 256
E.W.	1450	80	185	1 : 128
P.S.	690	40	215	< 1 : 8†
R.H.	340	40	40	< 1 : 8
G.L.	340	7	50	< 1 : 8

* Normal serum immunoglobulins: 1158 ± SD 305 mg/100 ml IgG; 99 ± SD 27 mg/100 ml IgM; and 200 ± SD 64 mg/100 ml IgA.

† Complement fixing antibody titre in this serum was < 1 : 8 but patient produced 1 : 16 titre 1 year after virus infection detected by isolation.

Table 1 details the concentrations of serum IgG, IgM, and IgA in samples from four patients whose peak CF antibody responses were recorded in the same sera. Sera was also collected 1–2 months after CMV was first isolated from the two patients who never responded to their CMV infection with CF antibody and from the one patient who responded only 1 year later. This 1–2-month time period was selected since all other patients had shown significant increases in CF antibody titres by this time after virus isolation. Table 1 also details the corresponding peak CF antibody titres in these sera.

Sera from two (patients R.H. and G.L.) of the seven patients were shown to have immunoglobulin (IgG, IgM, and IgA) levels greater than two standard deviations below the mean (Table 1). Neither of these patients produced detectable CF antibody and both died of mixed bacterial and viral infections. Serum immunoglobulins from two patients (R.S. and E.B.) were greater than two standard deviations above the mean and both of these patients correspondingly produced extremely high CF antibody titres in response to their CMV

infections. High CF antibody responses were also detected in sera from two patients (K.L. and E.W.) whose serum immunoglobulins were within one standard deviation of the mean. One patient (P.S.), with moderately low (about two standard deviations below mean) serum immunoglobulins produced only a relatively low CF antibody titre 1 year after the CMV infection was first detected.

One consistent and interesting observation was that the CF antibody responses of these immunosuppressed patients were usually ten to a hundred times higher than those found in non-immunosuppressed patients. Others have reported (Stern & Elek, 1965) that normal patients infected with CMV respond with titres of 1:16 to 1:64. We have observed rises in antibody titre of this same magnitude in non-immunosuppressed patients infected with CMV. Of the forty-two immunosuppressed patients who produced CF antibody to CMV, forty (95%) had antibody titres of 1:64 or greater and ten (24%) had titres of 1:1024 or greater.

TABLE 2. Inverse relationship between average micro-PHA response and peak anti-cytomegalovirus complement fixing antibody titre. The high and low micro-PHA response were discarded and the remaining results averaged. Chi square evaluation showed the inverse relationship valid at the significance level of $P < 0{\cdot}05$.

Peak anti-cytomegalovirus complement fixing antibody titres	Average micro-PHA response	
	Less than 1400 cpm	Greater than 1400 cpm
$\geqq$1:256	17	6
$\leqq$1:128	9	10

It was clear that the four patients who died during the first 3 months following transplantation had low micro-PHA responses and did not respond with CF antibody to their CMV infection. All other patients developed at least minimal micro-PHA responses and produced CF antibodies in response to CMV infection. Furthermore, an inverse relationship between average level of micro-PHA responsiveness and the peak CF antibody response was observed. Patients with higher than average micro-PHA responses had lower CF antibody titre than did patients with lower micro-PHA responses (Table 2). Accordingly, only two of the ten patients with CF antibody titres of 1:1024 or greater had a micro-PHA response of more than 1400 cpm.

DISCUSSION

Immunosuppressive therapy of some renal transplant recipients can result in severe and fatal virus infections (Craighead, Hanshaw & Carpenter, 1967; Fine *et al.*, 1972; Rifkind, Goodman & Hill, 1967). Since these few patients are treated according to regimens which are well tolerated by other patients, they appear to be more sensitive to immunosuppressive treatment than are most patients. In this study, tests which reflect humoral and cell-mediated

immunity were used to monitor immune responsiveness in these patients, and an attempt was then made to correlate these findings with the clinical courses of the patients.

Since most of the study patients with CMV infections produced at least normal levels of CF antibodies in response to their infection, humoral immune responses to CMV was not adversely affected by the immunosuppressive drugs. These conclusions are similar to those reached by others (Armstrong *et al.*, 1971; Craighead (1969); Huraux *et al.*, 1972; Prince *et al.*, 1971). A notable exception to this generalization is represented by a small group of patients who were so severely immunosuppressed that their cells could not respond to PHA in autologous plasma and they could not respond to their own CMV infections with CF antibody. Immunoglobulin levels were determined on two of these patients and were found to be more than two standard deviations below the mean. All four patients died whereas patients who developed CF antibodies against CMV and who ultimately showed some degree of PHA responsiveness almost all survived.

As opposed to humoral immune response, micro-PHA responsiveness was uniformly depressed by the immunosuppressive drugs and by uraemia. Since the micro-PHA test measures responsiveness in whole heparinized blood, both the thymus-derived lymphocytic response and serum factors which affect the response are assayed simultaneously. The technique does not discriminate between the effect of a reduced number of circulating T cells, a depressed response of the T cells to PHA, and serum factors (antibodies, immunosuppressive drugs) which might depress PHA responses. The technique was deliberately chosen to assess more precisely total *in vivo* responsiveness of the T-cell population by a single *in vitro* test. Our study, as well as those of many others, have shown that isolated, repeatedly washed lymphocytes from immunosuppressed patients respond normally to PHA when tested in pooled normal plasma. Lymphocytes from uraemic patients have also been shown to respond normally to PHA when tested in normal plasma (Daniels *et al.*, 1971). The micro-PHA test designed by Park & Good (1972) appears to detect changes attributable to immunosuppression that reflect an overall influence and most strikingly demonstrates that the lymphocytes of patients prone to lethal infection cannot respond to PHA in autologous blood. Since these lymphocytes appear to become sensitized to donor organ antigens during immunosuppression, and respond to mitogens in normal sera, the autologous sera appears to interfere with proliferation and function of these lymphocytes.

The discrepancy between the micro- and macro-PHA responses indicates that the degree of immunosuppression is probably dependent on the concentration of immunosuppressive agents in the peripheral blood of these patients. If this is the case, then the patients who were severely immunosuppressed probably have higher concentrations of these immunosuppressive agents even though they are receiving the same dosages as are other patients. One possible explanation is that these patients cannot clear the drugs as rapidly as can other patients, that they accumulate more of the repressive drugs and therefore become more immunosuppressed. The micro-PHA test appears to offer a method for quantifying the effective serum factors in the blood of such patients.

Others (Craighead, 1969; Huraux *et al.*, 1972; Prince *et al.*, 1971) have noted that immunosuppressed renal transplant patients produced higher CF antibody responses to their CMV infections than did normal persons. Our study corroborates and extends these observations. When patients with severe deficits of both humoral and cell-mediated immunity are excepted, there appears to be an inverse relationship between the degree of suppression of micro-PHA responsiveness and the CF antibody titre. Studies of experimental animals have demon-

strated that adult thymectomized, congenitally athymic (nude), and ALG-treated normal mice respond to thymus-independent antigens (antigens which do not require the helper function of T cells for antibody response) with higher than normal antibody titres (Baker *et al.*, 1973; Kerbel & Eidinger, 1972). Results from these studies have been interpreted as indicating that there is a T-cell sub-population which exerts a negative effect on antibody response. Both ALG-treated normal mice and nude mice apparently lack the suppressor T cells which allow for higher than normal antibody responses. It is equally probable that normal T-cell immunity to such antigens contributes to elimination of these antigens and that suppression of this modality by cytotoxic drugs results in a relative lack of this consequence. This would lead to greater stimulation of the responsive B-cell population yielding greater production of antibody under the circumstances.

The immunosuppressive treatment of our patients resulted in markedly higher antibody responses to CMV which we would conclude is probably a thymus-independent antigen. Suppression of T-cell function, as detected by the micro-PHA test, then would be expected to lead to suppression of the suppressor cells or the supressor function of T-cell immunity and higher antibody response to CMV. These high antibody responses, therefore, could represent a B-cell compensation for the relative lack of T-cell immunity reflected by the depressed micro-PHA response.

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