Clin. exp. Immunol. (1977) **27**, 454–463.

E-rosette forming cell numbers in the blood of human renal allograft recipients

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(*Received* 14 *September* 1976)

SUMMARY

The incidence of circulating T lymphocytes (E_T-RFC) and a sub-population of T lymphocytes (E_E-RFC) were monitored in the blood of seventy-one cadaver renal allograft recipients for the first 2 months after transplantation. In patients with uneventful post-operative courses, the incidence of both E_T-RFC and E_E-RFC fell promptly upon initiation of immunosuppression returning approximately to pre-operative levels 3–5 weeks after operation; the fall in cell numbers was greatest in those patients receiving adjunct ALG therapy. With the onset of an acute rejection episode, the E_E-RFC level rose quickly eventually exceeding the pre-operative level; in 88% (thirty episodes) of cases this rise occurred 1–6 days before clinical diagnosis of rejection and in 12% of cases on the same day as clinical diagnosis. The incidence of E_T-RFC rose in conjunction with some cases of acute rejection but remained unchanged in other cases. It is suggested that measurement of the incidence of E_E-RFC in blood is valuable in predicting the onset of acute rejection and for the differential diagnosis of acute rejection and ischaemic renal damage.

INTRODUCTION

Assessment of immune status remains a major difficulty in management of organ transplant recipients. Inadequate immune suppression allows rejection of the graft while excessive suppression increases susceptibility to pathogens.

At present, immune suppressive management of renal transplant recipients is based on biochemical tests of allograft function. Such tests do not distinguish between immunological and non-immunological causes of renal damage, and give no indication of the degree of immune suppression of patients.

Tests which attempts to measure immune competence include cutaneous hypersensitivity reactions to soluble donor-type antigens (Kahan *et al.*, 1973), inhibition of leucocyte migration with donor-specific antigens (Smith *et al.*, 1969; Richmond, Doak & North, 1973; Wood *et al.*, 1973) and aggregation by recipient lymphocytes to donor-type kidney cells or fibroblasts in tissue culture (Kahan *et al.*, 1974). All these assays successfully detect increasing immunologic reactivity but are of limited clinical use as some do not give results quickly enough, others present technical difficulties in testing large numbers of patients frequently and others are insufficiently quantitative.

Recent studies suggest that cellular immunity is reflected by levels of thymus-dependent lymphocytes (T cells) in the peripheral blood. Patients with reduced cellular immune responsiveness in a variety of disease processes have reduced T-cell levels in their blood (Cohnen *et al.*, 1973; Wybran & Fudenberg, 1973; Wybran *et al.*, 1973b; Yata *et al.*, 1973; Catalona, Potvin & Chretien, 1974b). Further, relative and absolute numbers of T cells in peripheral blood decrease following administration of immunosuppressive drugs (Bettcher, Mix & Dossetor, 1973; Olweny & Levanthal, 1973; Fauci & Dale, 1974; Bishop *et al.*, 1975) or irradiation therapy (Stjernsward *et al.*, 1972; Catalona, Potvin & Chretein,

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1974a) and increased levels of RFC have been reported in human transplant recipients during rejection episodes (Cullum *et al.*, 1972; Bewick *et al.*, 1972).

Estimation of T-cell numbers in peripheral blood is a rapid and simple procedure based on the ability of human T cells to form E rosettes with sheep red blood cells. Two methods which detect greatly different levels of E-rosette-forming cells (E-RFC) in human blood are described in the literature. With one technique (Jondal, Holm & Wigzell, 1972) between 55–88% of peripheral blood lymphocytes (PBL) form E rosettes, these proportions approximating the total number of T cells in blood. With the other (Froland, 1972; Wybran, Carr & Fudenberg, 1972), between 15 and 40% of PBL form rosettes, and these cells probably represent a sub-population of T cells.

In this study we followed the levels of both types of E-RFC in the peripheral blood of cadaveric donor renal allograft recipients for periods up to 2 months after operation. For each patient the course of E-RFC numbers post-operatively was compared with allograft function. We were interested to see if these assays could be used to determine the cellular immunologic reactivity of renal allograft recipients, with particular reference to early diagnosis of acute rejection, differentiation of immunological and non-immunological causes of renal failure and detection of excessive immune suppression.

MATERIALS AND METHODS

Rosette test. Venous blood was collected into preservative-free heparin (20 iu/ml of blood) and immediately chilled to 5°C. Samples reached the laboratory 60–90 min after collection and were maintained at 5°C for this period. Lymphocytes were separated by density-gradient centrifugation over Ficoll–Hypaque mixture, washed twice and resuspended in Hanks's balanced salt solution (HBSS) containing 0·2% bovine serum albumin. Human lymphocytes (3×10^6) cells in 0·75 ml of HBSS were mixed with 0·25 ml of fresh 5% sheep red blood cell (SRBC) solution, resulting in a mixture containing approximately twenty-five SRBC per human lymphocyte.

Methods to obtain rosettes were: 1. *'Early' E rosettes (E_E-RFC).* The cell suspension was centrifuged at 200 *g* for 5 min followed by resuspension on a rotary disc at 6 rev/min for 10 min at room temperature. A drop of the suspension was then examined immediately in a haemocytometer chamber.

2. *'Total' E rosettes (E_T-RFC).* The cell suspension was centrifuged at 200 *g* for 5 min and then incubated at 5°C for 4 hr. The cell button was then gently resuspended with a Pasteur pipette and examined in a haemocytometer chamber.

For the two types of rosettes, approximately 1000 lymphocytes were counted and the percentage of lymphocytes that formed rosettes calculated. A rosette was defined as a lymphocyte with at least three adhering SRBC.

Patients. Control patients. The incidence of E_E-RFC and E_T-RFC were measured in the peripheral blood of fifty-four healthy blood donors aged between 20 and 45 years.

Allograft recipients. The study involved seventy-one patients who underwent cadaveric renal allotransplantation operations. Patients were adults of either sex who had developed chronic renal failure as a result of a variety of renal diseases. Before transplantation all patients were maintained by haemodialysis. Post-operative management of patients in this transplant programme is described in detail elsewhere (Sheil *et al.*, 1971). Briefly, immune suppression was achieved with azathioprine and prednisone administration daily; sixty of the seventy-one patients received additional ALG therapy for between 4 and 12 days after operation. Rejection crises were treated by increased prednisone dosage. Rejection was diagnosed clinically or, in those patients with good renal function, on the basis of rises in the serum creatinine (SCr) level of 0·3 mg per 100 ml of blood on consecutive days of testing in the absence of surgical or infective complications.

E_E-RFC numbers were monitored in all seventy-one patients; E_T-RFC numbers were additionally followed in eleven patients. The numbers of both E_E-RFC and E_T-RFC in blood were estimated before operation on the day of transplantation and then between three and five times weekly for periods up to 2 months. On each occasion blood for testing was collected before administration of daily immune suppressive therapy.

Patients were categorized into three groups according to graft function following transplantation:

Group A. Those with good early graft function and no later rejection episodes. There were nine patients in this group.

Group B. Those patients with good early graft function and later rejection episodes. There were thirty patients in this group. Twenty-eight patients had a total of thirty-four 'definite' acute rejection episodes within the first 7 weeks after transplantation. In each case there was significant deterioration of renal function in the absence of any other recognizable complication; in many of these cases, renal biopsy substantiated rejection. Two patients had 'doubtful' acute rejection episodes. In both cases SCr levels fell uneventfully after transplantation but plateaued suddenly 1 week after operation. High dose steroid therapy was instigated and the SCr levels promptly continued to fall.

Group C. Those patients with protracted poor graft function from time of transplantation. There were thirty-two patients in this group. Analysis of this group is more difficult as delayed renal function can be due to a variety of causes and all patients were treated empirically with anti-rejection therapy approximately once each week. In twenty-eight patients, delayed graft function was attributed primarily to tubular necrosis following prolonged ischaemia, and these grafts ulti-

mately functioned well. In the other four patients poor graft function was due to technical complications (arterial thrombosis (two), ureteric obstruction (one), graft infection (one)) requiring eventual nephrectomy.

RESULTS

Control patients

The incidence of E_E-RFC and E_T-RFC in the peripheral blood of normal, healthy individuals is shown in Table 1. In fifty-four adults between 7 and 41% (mean 19·9%) of PBL formed E_E-rosettes; between 53 and 85% (mean 72·5%) of PBL formed E_T rosettes. With three subjects, repeated assays of both E_E-RFC and E_T-RFC numbers in blood over a 4–6-month period showed day-to-day variations in both kinds of between 0–6%.

Allograft recipients

'Early' (E_E-RFC) numbers. The level of E_E-RFC in the blood of patients immediately prior to transplantation was similar to that in normal, healthy individuals (Table 1). While the incidence of E_E-RFC remained relatively constant within control individuals, in groups A, B and C patients day-to-day

TABLE 1. The incidence of E_E-RFC and E_T-RFC in the blood of normal individuals and renal allograft recipients immediately before transplantation

	No. of subjects	Range* (%)	Mean (%)
'Early' (E_E) RFC			
Normal individuals	54	7–41	19·9
Transplant patients	30	4–35	18·2
'Total' (E_T) RFC			
Normal individuals	54	53–85	72·5
Transplant patients	11	60–86	71·1

* Percentage of PBL that form rosettes with SRBC.

variations up to 16% (i.e. 16 RFC per 100 PBL counted) occurred in the first few weeks following transplantation, but a sudden rise was not regarded as significant unless the E_E-RFC level eventually exceeded the pre-operative level.

1. *Group A patients.* In five of the nine patients in this group post-operative courses of E_E-RFC numbers were similar; all five patients received ALG therapy. In each, the incidence of E_E-RFC was markedly reduced for the first few days after transplantation, remaining suppressed for the duration of ALG therapy, then rising sharply upon completion of therapy, though in no case to levels exceeding those recorded before operation. Following the rise after completion of ALG therapy, E_E-RFC levels fell moderately in each case but then gradually rose to approximately pre-operative levels from 20 to 30 days after transplantation. The post-operative course of E_E-RFC numbers in the blood of one of the five patients (no. 1) is presented graphically in Fig. 1. In another two patients, who also received ALG therapy, the fall in the incidence of E_E-RFC was less pronounced; the post-operative course of E_E-RFC numbers in one of these two patients is presented in Fig. 1. The ALG preparations used in the former five patients were potent (rosette-inhibition values of 1:16,000) while those preparations used in the latter two patients were less so (rosette inhibition values of 1:1000).

In one other patient there was a rapid increase in the number of RFC immediately after transplantation, followed by a sharp fall. The only unusual feature of this patient was a severe reaction to the initial injection of ALG; this was the patient's second graft and he had received previous ALG therapy.

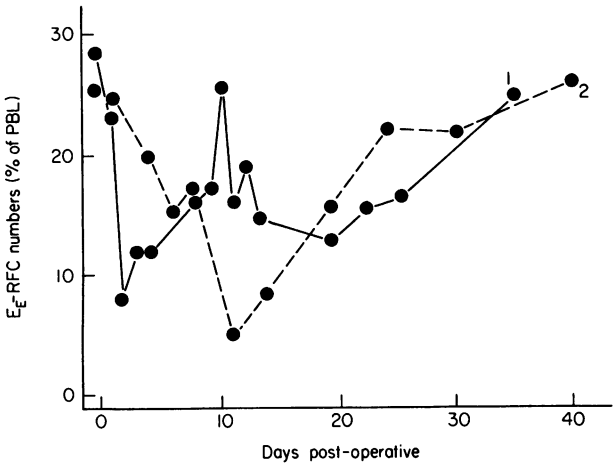

FIG. 1. E_E-RFC numbers in the blood of two group A patients. These patients had good early graft function following transplantation and no later rejection episodes. Both patients received adjunct ALG therapy; patient no. 1 was treated with an ALG preparation of high potency and patient no. 2 an ALG preparation of low potency. (●——●) Patient no. 1; (●- - -●) patient no. 2.

The remaining patient in this group received no adjunct ALG therapy and in this patient the incidence of E_E-RFC fell gradually from the time of transplantation, returning approximately to the pre-operative level 5 weeks after transplantation.

2. *Group B patients.* In each of the twenty-three 'definite' acute rejection episodes in group B patients, there was a substantial rise in the incidence of E_E-RFC in blood preceding or concomitant with clinical diagnosis of rejection. In those patients where the rejection episode occurred within the first week following transplantation, E_E-RFC numbers usually rose from the time of transplantation (Fig. 2). In other patients with later-developing acute rejection crises, E_E-RFC numbers fell following transplantation as with group A patients but then rose sharply in association with the rejection episode (Fig. 3). In each case the rise in the incidence of E_E-RFC was abrupt, usually reaching a peak level

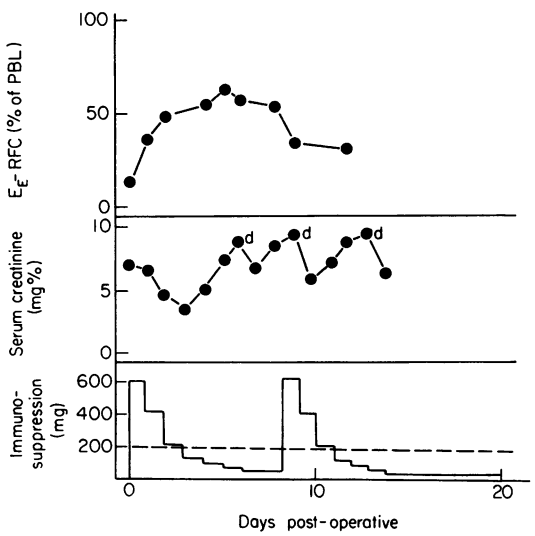

FIG. 2. Serum creatinine levels and E_E-RFC numbers in a patient with an early-developing acute rejection episode. There is a rise in the serum creatinine level 4 days after transplantation; E_E-RFC numbers rose from the time of transplantation. Imuran (- - - -); prednisone (——); haemodialysis therapy (d).

within 2–3 days and in all cases, peak E_E-RFC levels during rejection crises (range 20–67% of PBL; mean 37·7%) exceeded pre-operative levels (range 4–35%; mean 18·8%).

The temporal relationship between increase of E_E-RFC levels in excess of pre-operative levels (thereafter termed a significant rise) and increase of SCr levels is shown in Table 2. In thirty cases

TABLE 2. Increase in the incidence of E_E-RFC in the blood of Group B patients in association with acute rejection episodes. For each patient, the day there was a significant rise in the incidence of E_E-RFC is compared with the day the SCr level rose

Rise in E_E-RFC level in relation to rise in SCr (days)	No. of rejection episodes
−6	3
−5	2
−4	6
−3	8
−2	4
−1	7
Same day	4
Total	34

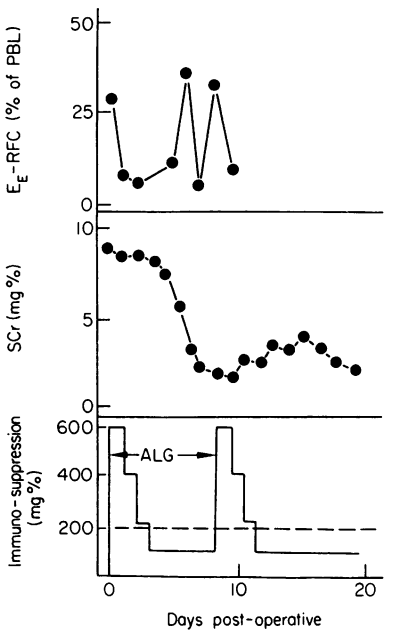

FIG. 3. Incidence of E_E-RFC in a patient with an acute rejection episode. The rise in E_E-RFC numbers precedes the rise in serum creatinine level by 4 days. Note the sharp fall and then further rise in E_E-RFC numbers following the initial rise. Imuran (- - - -); Prednisone (——).

(88%) a significant rise in the incidence of E_E-RFC preceded the rise in SCr level by an interval of 1–6 days. In four cases, pre-operative E_E-RFC levels were exceeded on the same day that SCr levels increased. No false positive rises in the incidence of E_E-RFC were observed in these patients, each significant rise being associated with clinically recognizable rejection.

In eleven (32%) of the thirty-four patients with 'definite' rejection, immediately following the initial significant rise in the incidence of E_E-RFC, there was a sudden profound fall and subsequent rise in the E_E-RFC level again (Fig. 3). In nine of the eleven cases this abrupt fall occurred before clinical diagnosis and treatment of rejection so that this phenomenon could not be related to increased immunosuppression. In all patients, the incidence of E_E-RFC was much reduced 24 hr after increased prednisone dosage for rejection.

In the two cases of clinically 'doubtful' acute rejection, there was a significant rise in the incidence of E_E-RFC preceding plateauing of SCr levels, with RFC levels subsequently falling following empirical treatment for rejection.

3. *Group C patients.* In twenty-eight patients in group C, delayed graft function was attributed primarily to tubular necrosis and in these patients E_E-RFC levels following transplantation were variable. In thirteen E_E-RFC levels followed a course similar to that observed in most group A patients, with a fall in E_E-RFC levels shortly after transplantation returning approximately to pre-operative levels after 3–4 weeks. Other than moderate day-to-day variations in E_E-RFC numbers as observed in some patients in groups A and B, and sharp falls in RFC numbers following empirical treatment for rejection, these

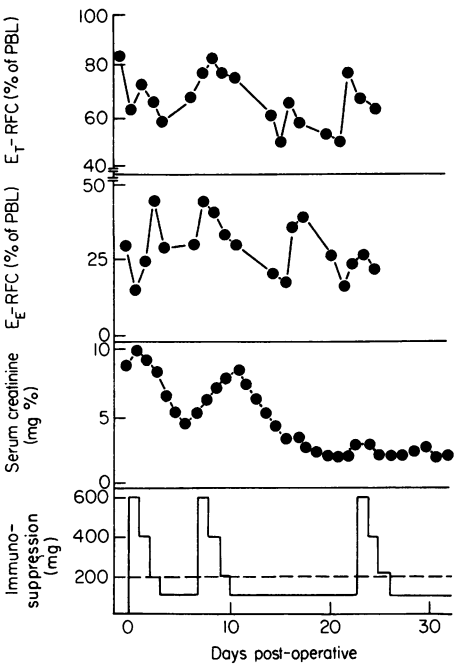

FIG. 4. Patient with two episodes of acute allograft rejection indicated by rises in the SCr level on days 7 and 23 respectively after transplantation. With each episode, there is an increase in the incidence of E_E-RFC preceding the rise in SCr level; E_T-RFC levels increased simultaneously with rising SCr levels, but in each case the peak E_T-RFC level did not exceed the preoperative level. Imuran (- - - -); prednisone (——).

patients showed no significant rises in E_E-RFC numbers as were observed in group B patients in association with rejection. Graft biopsies were performed in three of these thirteen patients during the oliguric phase—in two grafts there was mild, patchy cellular infiltration of the renal parenchyma and in one no evidence of rejection.

In fifteen patients, the incidence of E_E-RFC increased significantly at one or more times following transplantation in a manner similar to that observed in group B patients undergoing acute graft rejection. In five, the rise in incidence of E_E-RFC preceded by several days clinical diagnosis of rejection because of fever and graft tenderness. Graft biopsies were performed in four other patients—in each case microscopy revealed tubular necrosis and moderate diffuse cellular infiltration of graft tissue.

In the remaining four patients in group C whose grafts failed for technical reasons, E_E-RFC levels fell following transplantation and were low at the time of graft removal; the four grafts were removed 4–17 days following transplantation and in each case microscopic examination revealed little evidence of rejection.

'Total' (E_T) RFC numbers. The incidence of E_T-RFC was followed in six group B patients and five group C patients. The level of E_T-RFC in the blood of these eleven patients immediately prior to transplantation was similar to that in normal healthy individuals (Table 1). Immediately following transplantation, E_T-RFC numbers fell in seven patients but showed little change in the other four.

In the six group B patients, there were a total of eight rejection episodes. In two cases of first rejection and one of second, E_T-RFC levels increased significantly, to a level exceeding the pre-operative level, preceding increased SCr levels (6, 5 and 2 days respectively); in each case the rise in E_T-RFC paralleled the rise in E_E-RFC levels. In two other cases of rejection, E_T-RFC levels and SCr levels rose on the

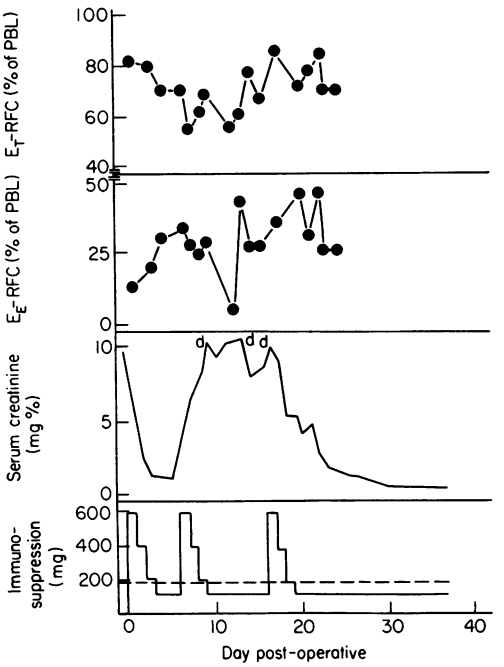

FIG. 5. Patient with acute allograft rejection with a rise in the level of serum creatinine 5 days after transplantation. The incidence of E_E-RFC rose from the time of transplantation while the incidence of E_T-RFC remained below pre-operative levels for 2 weeks after transplantation. Imuran (- - - -); prednisone (——); haemodialysis therapy (d).

same day, but in each case peak E_T-RFC levels during the rejection episode did not exceed the pre-operative level (Fig. 4); in three other cases of rejection, there was no rise in E_T-RFC levels though in both E_E-RFC rose significantly. E_E-RFC and E_T-RFC levels in one of these last patients are shown in Fig. (5).

In the five patients in group C, two patients had E_T-RFC levels which, apart from moderate day-to-day variations, remained unchanged following transplantation; in one patient no rejection episode was suspected or detected but in the other a rejection episode was suspected on clinical grounds and at this time the E_E-RFC level showed a sudden increase. In the other three patients, E_T-RFC levels rose and eventually exceeded the pre-operative level at some stage following transplantation in parallel with a significant increase in E_E-RFC levels; in two of these three cases the rise in RFC numbers was shortly followed by strong clinical evidence of rejection. In the third case there was no concominant rise in E_E-RFC level and no clinical suggestion of rejection.

Follow-up studies

E_E-RFC and E_T-RFC levels were followed in six patients following their discharge from hospital; patients were tested at 1–3-month intervals for up to 2 years after transplantation. All six patients had good kidney function and no major complications during the period of testing. In each case, both E_E-RFC and E_T-RFC levels approximated the pre-operative values and remained at these levels with only minor variations.

Seven other patients were tested for a short period upon re-presentation at hospital for a variety of infective complications; in each case kidney function was good. In all patients at the time of discharge from hospital following transplantation E_E-RFC and E_T-RFC levels approximated the pre-operative levels. Two patients with severe oral fungal (Monilia) infection had much elevated E_E-RFC levels (63% and 69% of PBL respectively); E_T-RFC levels were normal in both cases. Two other patients had bacterial infections (pneumococcal lung infection and streptococcal urinary tract infection respectively); in both cases E_E-RFC and E_T-RFC levels were normal. Three other patients had severe Herpes Simplex infection; in one the E_E-RFC level was marginally reduced below the pre-operative value and in the other two the E_E-RFC levels were much reduced, while in all three E_T-RFC levels approximated pre-operative values.

DISCUSSION

The phenomenon of rosette formation by human lymphocytes with SRBC is poorly understood, albeit it is clear that RFC are thymus-dependant cells (T-lymphocytes) (Froland, 1972; Jondal *et al.*, 1972; Bach, 1973). Equally uncertain is the reason for the differential avidity of human T-lymphocytes for SRBC. The small proportion of lymphocytes which form rosettes immediately after contact with SRBC (E_E-RFC) give rise to characteristically stable structures, the SRBC being firmly bound to the central lymphocyte. If the lymphocytes are allowed to remain undisturbed in close contact with SRBC, the incidence of lymphocytes forming E rosettes increases with time until a maximum number of RFC is reached after approximately 4 hr incubation at either 4°C or 20°C (Kelly, 1972). A distinctive feature of these later-forming rosettes is that they are much less stable structures than the E_E-rosettes, and are readily disrupted by agitation. The later-forming E rosettes, however, can be made stable either by incubating the lymphocyte-SRBC mixture in the presence of human serum (Wybran, Carr & Fudenberg, 1973a) or by pretreating the SRBC with neuraminidase (Galili & Schlesinger, 1974) or a sulphydryl reagent (Kaplan & Clark, 1974).

Wybran and co-workers have suggested that the stronger-binding 'early' RFC represent a subpopulation(s) of T lymphocytes involved in the active aspects of cellular immunity and are thus a more sensitive measure than the total T-cell level in blood of the state of activity or competence of cellular immune responsiveness (Wybran & Fudenberg, 1971; Wybran *et al.*, 1973b). The results of this study lend support to this hypothesis with E_E-RFC numbers proving to be a far more reliable index of the effectiveness of immune suppression in the early days following transplant operations than E_T-RFC numbers.

In renal transplant recipients with functioning renal grafts and no clinical evidence of rejection, E_E-RFC levels were suppressed following transplantation to a variable degree, subsequently returning approximately to pre-operative levels from 3–5 weeks after transplantation. In follow-up studies of several patients examined up to 2 years after transplantation, E_E-RFC levels subsequently showed little variation in the absence of complications.

It would appear that adequate immune suppression does not necessarily require profound depression of E_E-RFC numbers. Some patients with only slight suppression of E_E-RFC levels had good graft function and showed no evidence of rejection while other patients whose E_E-RFC levels were profoundly suppressed after transplantation had acute rejection crises soon afterwards. It would therefore seem unwise to rely on E_E-RFC levels to adjust the amounts of immunosuppressive agents given, since for different patients the degree of suppression required will vary, being related to the degree of antigenic disparity between donor and recipient. In this respect, other assays which measure specific

activity of recipient lymphocytes to donor or donor-type cells or antigen (Richmond, Doak & North, 1973; Kahan *et al.*, 1974) may be superior.

In those patients with rejection crises, E_E-RFC levels increased sharply, eventually exceeding pre-operative values; in almost all patients (88%) this rise in E_E-RFC levels preceded the rise in SCr levels, the parameter currently used by the majority of groups as the most sensitive index of rejection. That is, as a predictive assay for acute rejection the results of the study were encouraging. It should be noted that in the four (12%) patients in whom E_E-RFC levels did not rise before rejection was diagnosed on the basis of clinical or biochemical evidence, E_E-RFC were monitored, as in all patients, only three times weekly. In view of the curious phenomenon of a sharp rise, fall and then rise again in E_E-RFC numbers detected in some patients with acute rejection episodes it would seem advisable to test patients daily to ensure that the initial rise in RFC numbers is detected. The sudden fall in E_E-RFC numbers after the initial rise occurred mostly before doses of immune suppressive agents were increased for the rejection episodes and is difficult to explain. As the total T-cell level (E_T-RFC) in the blood was often within normal limits or even elevated at the time of the fall in E_E-RFC numbers it is unlikely that it represents a transitory absence or removal of these cells from the blood. Rather it indicates a temporary inability to firmly bind SRBC.

It seems that ALG therapy affects E_E-RFC levels. Those patients in whom E_E-RFC levels were profoundly reduced post-operatively received adjunct ALG therapy whereas in those patients not receiving ALG, E_E-RFC levels post-operatively were generally only slightly reduced. Further, ALG batches with high rosette-inhibiting values therapy caused a sharp decrease in E_E-RFC numbers and E_E-RFC numbers were found to increase sharply within 24 hr of completion of ALG therapy. Bishop *et al.* (1975) similarly observed more effective depletion of circulating E-RFC in renal transplant patients with adjunct ALG therapy. However, it remains to be shown whether ALG effectively removes RFC from blood or whether *in vivo* 'coating' of RFC with ALG subsequently interferes with *in vitro* binding to SRBC.

The effect of infective complications on E_E-RFC levels is difficult to assess in view of the small number of patients involved, but the findings of this study are in substantial agreement with those of Wybran & Fudenberg (1973) who examined E_E-RFC levels in the blood of a larger number of patients with various viral, bacterial and mycotic infections. E_E-RFC levels were elevated in two patients with fungal infection, depressed in two patients with viral infection and unchanged in two patients with bacterial infection and one patient with viral infection.

Post-operative management of transplant patients is often necessarily complex involving such factors as immunosuppressive agents, antibiotics, miscellaneous drugs, infective complications, blood transfusions, surgical procedures, and supportive measures such as haemodialysis. The total effect of these various parameters on the immune system, and more particularly, the level of E_E-RFC in blood, is not clear. Obviously the results of an assay such as that described here must be assessed objectively, taking into account the considerations mentioned above. Nevertheless the assay has many attractive features. It is simple to perform and read, requires only a small quantity of blood and the test is completed within 3 hr. Moreover, it provides three clear advantages. First, an early diagnosis of rejection, thereby increasing the effectiveness of anti-rejection therapy. Second, management of patients with delayed graft function due to tubular necrosis is simplified by reducing the need to treat empirically for rejection. Third, it helps distinguish between poor graft function due to acute rejection and that due to non-immunological causes, for example urinary obstruction, arterial thrombosis etc.

Finance for this study came from the Australian Kidney Foundation and the Post-Graduate Medical Foundation of the University of Sydney. One of us (G.E.K.) held an R.T. Hall Research Fellowship.

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