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INHIBITION OF LEUCOCYTE MIGRATION AS AN INDEX OF REJECTION IN RENAL TRANSPLANT RECIPIENTS

D. E. RICHMOND, P. B. DOAK AND J. D. K. NORTH

Department of Medicine, Auckland Hospital, Auckland, New Zealand

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SUMMARY

In a blind study, the peripheral blood leucocytes of fifteen recipients of cadaver kidney homografts have been checked at regular intervals for migration inhibition in the presence of antigen derived from donor spleen. Six of seven episodes of definite acute rejection were preceded or accompanied by migration inhibition, and four of seven probable acute rejection crisis were similarly accompanied by inhibition. No false positive tests were observed. The technique may be a useful adjunct to present methods of diagnosing acute homograft rejection.

INTRODUCTION

The early detection of a rejection crisis is important in the management of patients who have received renal transplants. At present the diagnosis of rejection is based primarily on evidence of deteriorating renal function accompanied by less specific physical findings such as fever, an increase in blood pressure, tenderness over the graft and changes in the urinary sediment. These criteria are non-specific in the sense that concurrent urinary tract infection, graft ischaemia and obstructive uropathy may all be associated with a similar constellation of symptoms and signs. Furthermore, it is apparent that a diagnosis depending on deteriorating graft function may be made too late, since in a proportion of cases, despite apparent reversal of the rejection crisis with anti-rejection treatment, graft function fails to return to pre-rejection levels.

It is reasonable to suppose, since graft rejection represents an immune response, that an immunological method of detecting early homograft rejection could be developed. Indeed, several techniques have been investigated (Austen & Russell, 1966; McDonald, 1966; Rubin *et al.*, 1964), but none seems to have been sufficiently reproducible, reliable or convenient to be of use as a routine predictive test of graft rejection.

In recent years the inhibition of migration of macrophages and lymphocytes by specific antigen has been used increasingly as an *in vitro* correlate of the state of cellular immunity in experimental animals (George & Vaughan, 1962; Bloom & Bennett, 1966; David, 1966)

and in humans (Thor *et al.*, 1968; Rocklin, Myers & David, 1970). Early studies used guinea-pig peritoneal macrophages, but the modification of Söborg & Bendixen (1967) using peripheral blood lymphocytes appears to be equally valid (Rosenberg & David, 1970) and reports of its use as a predictive test of kidney graft rejection have begun to appear in the literature (Smith *et al.*, 1969; Galanaud *et al.*, 1972). This paper reports our experience with migration inhibition of peripheral blood leucocytes in a series of fifteen renal homograft recipients.

PATIENTS

The patients were from our chronic haemodialysis and transplantation programme and had developed chronic renal failure as the result of a variety of renal diseases (Table 1). All received cadaver kidneys, the technique of grafting having been described elsewhere (Doak *et al.*, 1968). Their management post transplantation by the physicians responsible for

TABLE 1. Details of patients and kidney grafts

Patient	Sex	Age	Original diagonsis	Period followed (days)	Ultimate fate of graft
1	M	25	Chronic glomerulonephritis	114	Functioning graft
2	F	42	Analgesic nephropathy	88	Functioning graft
3	F	46	Chronic pyelonephritis	60	Rejected (patient died)
4	F	32	Chronic glomerulonephritis	30	Rejected
5	F	50	Polycystic kidneys	50	Functioning graft
6	M	50	Polycystic kidneys	68	Functioning graft
7	F	24	Renal cortical necrosis	107	Functioning graft
8	F	48	Chronic glomerulonephritis	53	Rejected (patient died)
9	M	39	Polycystic kidneys	113	Functioning graft
10	F	38	Malignant nephrosclerosis	134	Functioning graft
11	F	14	Chronic glomerulonephritis	20	Rejected
12	M	22	Chronic glomerulonephritis	110	Functioning
13	M	30	Chronic renal failure, cause unknown	50	Functioning
14	M	34	Analgesic nephropathy	46	Functioning
15	M	44	Chronic glomerulonephritis	30	Rejected

the transplant programme was independent of the migration inhibition study. The clinical status of the individual patients was not known to the investigators until cross-correlations were made at monthly intervals. The range for expected normal migration in the presence of spleen antigen was established by studying the degree of inhibition of migration of peripheral blood leucocytes from normal individuals. Twenty-eight studies were performed in triplicate using three different splenic extracts in both a high and a low concentration. From these results means and standard deviations were calculated and the normal range for high and low antigen concentrations taken as the mean ± 2 SD.

METHODS

Preparation of glassware

All glassware including glass base-plates, glass rings and capillary tubes was thoroughly washed and siliconized by immersion in a 1 : 100 solution of Siliclad (Clay Adams) for several minutes with agitation to ensure the escape of all air bubbles. Following thorough rinsing in distilled water, the glassware was dried at 110°C for 2 hr.

Preparation of antigen

A crude particulate preparation of donor spleen obtained under sterile conditions at the time of nephrectomy was used. The fresh spleens were coarsely minced, washed twice with Medium 199 and fragmented further by prolonged gentle agitation in fluted flask with sidearm on a reciprocating shaker. Erythrocytes were removed by exposure to 0·8% ammonium chloride solution for 8 min, the residue washed again, resuspended in Medium 199 and the cells were counted. A final concentration of 20×10^6 lymphocytes/ml in Medium 199 was prepared and the whole frozen in aliquots and stored at −20°C. Just before use each aliquot was rapidly thawed and disrupted by ultrasound (20,000 cycles, 150 W) in an ice bath using 30-second bursts for a total of 5 min. The final concentration of antigen used in the test chambers were equivalent to 3×10^6 cells/ml ('high dose') and 1×10^6 cells/ml ('low dose').

Migration inhibition was compared at two concentrations of antigen because Söborg (1968) has shown that at low levels of sensitization stimulation of migration may occur, to be followed by migration inhibition at higher levels of sensitization. Hence, whereas at high levels of sensitization inhibition occurs with both low and high concentration of antigen, in the intermediate range of sensitization the area of migration may fall within the normal range for any one concentration of antigen (Smith *et al.*, 1969) as the cell response changes from an inhibitory to a stimulating one. The use of high and low concentrations of antigen therefore assists in the identification of intermediate degrees of sensitization where the migration index is in the normal range for the high concentration of antigen, but elevated because of stimulation of migration at the low concentration. At still lower levels of sensitization, stimulation will be apparent for both high and low concentrations of antigen. By measuring the migration index with a low as well as a high concentration of antigen it is possible to place any particular value for the migration index on a theoretical curve of increasing sensitization (Fig. 1).

Leucocyte migration test

The procedure used was based on the technique of Söborg & Bendixen (1967) incorporating modifications suggested by Rosenberg & David (1970).

Once or twice weekly, 20 ml of blood were drawn from each patient into 500 units of sodium heparin in a sterile plastic syringe. After gentle mixing, the syringe was placed vertically in a rack and allowed to settle by gravity at room temperature. After settling of the red blood cells, all but the bottom 0·5 ml of leucocyte rich plasma was removed and transferred to a sterile plastic centrifuge tube. After cell counting the suspension was centrifuged at 150 *g* for 5 min at 4°C, and the cells washed three times in Medium 199. After the final wash, the leucocytes were resuspended in Medium 199 to which 10% horse serum had been added, to a final concentration of 3×10^6 lymphocytes/100 μl of medium.

A 1-ml syringe was used to fill six to nine previously siliconed and sterilized glass capillary

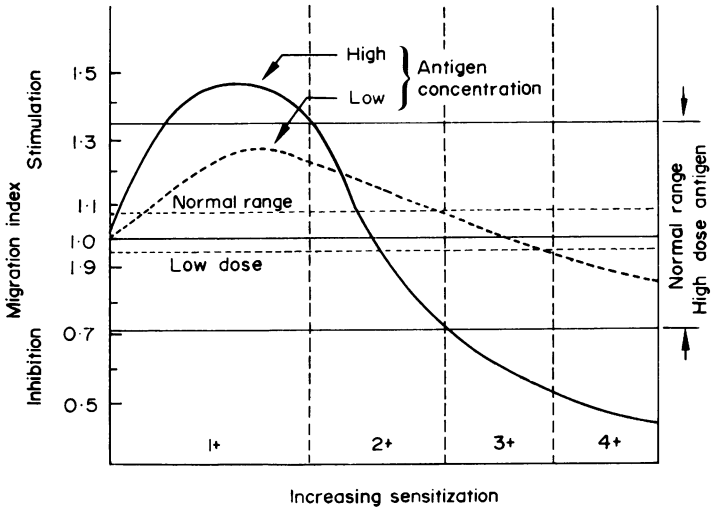

FIG. 1. Diagrammatic representation of the changes in migration index occurring with increasing sensitization.

tubes of 1 mm internal diameter. One end of each was heat-sealed. After centrifugation at 350 *g* for 5 min the tubes were cut just below the cell-fluid interfaces, and the portions containing the leucocytes anchored inside small circular glass chambers. The latter had been previously prepared by using silicone high vacuum grease (Dow Corning) to seal 0·5 mm deep × 1·5 mm internal diameter glass rings to a glass base plate.

The tests were set up in triplicate. Control chambers were filled with standard medium (Medium 199 with 10% horse serum), and test chambers with standard medium containing antigen in either high or low concentration.

The chambers were sealed with coverslips so that all air was excluded, and incubated lying flat for 18 hr at 37°C. The migratory area was then projected onto a screen and the outline traced on transparent paper. The area of migration was measured with a planimeter. The ratio of the area of migration in the presence of antigen to that in the absence of antigen was calculated and the result expressed in terms of a migration index.

$$\text{Migration index} = \frac{\text{Area of migration in presence of antigen}}{\text{Area of migration in control chambers}}.$$

The migration indices were recorded and at monthly intervals plotted in graphical form together with the daily dose of steroid (Prednisone), azathioprine (Imuran) and levels of serum creatinine and creatinine clearance. Also noted were the physicians comments in retrospect on the cause and outcome of events during the month which had been treated as instances of acute graft injection.

RESULTS

Studies in normal subjects

From these studies a range of normal migration indices (mean ± 2 SD) was calculated

for both the high and low antigen concentrations. For the high dose antigen the range was 0·71–1·33; for the low dose 0·95–1·07. Migration indices greater than 1·33 and 1·07 for high and low dose antigen respectively were regarded as evidence of stimulated migration, whilst indices lower than 0·71 and 0·95 were regarded as evidence of migration inhibition. In assessing the place for each result on the theoretical curve of increasing sensitization only those values in the arbitrary 3+ and 4+ range (Fig. 1) were regarded as unequivocal evidence of increased sensitization.

Studies in kidney transplant recipients

A summary of the clinical details pertaining to the fifteen patients is given in Table 1. The original diagnoses cover a wide range of renal disease. Eleven of the patients have functioning grafts, and in four the graft was rejected. Two patients have died. There were twenty incidents treated originally as acute rejection crises.

TABLE 2. Analysis of results

	Definite acute rejection	Probable acute rejection	False alarm	Chronic rejection	Migration inhibition without rejection
Total in group	7	7	5	1	1
Number and % showing early inhibition	5 (71)	3 (43)	0	1 (100)	
Number and % showing late inhibition	1 (14)	1 (14)	0	0	
Number and % showing no inhibition	1 (14)	3 (43)	5 (100)	0	

In Table 2 the results are analysed in terms of 'definite' acute rejection episodes, 'probable acute' rejection episodes, false alarms, chronic rejection and migration inhibition without clinical evidence of rejection. 'Definite acute' rejection episodes were those in which there was significant deterioration of renal function in the absence of any other recognizable complication, which reversed in response to increased immunosuppressive treatment. In many of these cases renal biopsy showed severe infiltration of the renal parenchyma by cells of the lymphocytic series.

A category of 'probable acute' rejection was included to cover those instances in which some doubt existed as to the role of acute rejection in the total clinical picture. For example one such case was complicated by a ruptured ureter, although biopsy of the graft revealed moderate cellular infiltrate. In two instances the long delay in onset of graft rejection prompted high dose steroid therapy resulting in rapid development of graft function. In such cases it is not possible to state on clinical grounds whether or not graft rejection was responsible for delayed graft function.

A 'false alarm' consisted of a deterioration of renal function which at first appeared to be due to acute rejection and later proved to have been due to some other complication, e.g. ruptured ureter or graft ischaemia.

There were seven episodes of definite acute rejection. Five of these (71%) were preceded

by persistent migration inhibition, in one case beginning 30 days before the clinical manifestations appeared. In a sixth, migration inhibition was evident for the first time the day after rejection was diagnosed and continued for 10 days thereafter. In the seventh, migration inhibition was not detected. Thus 86% of definite acute rejection episodes were preceded or accompanied by migration inhibition.

Three patterns of migration inhibition are illustrated in Figs 2–4.

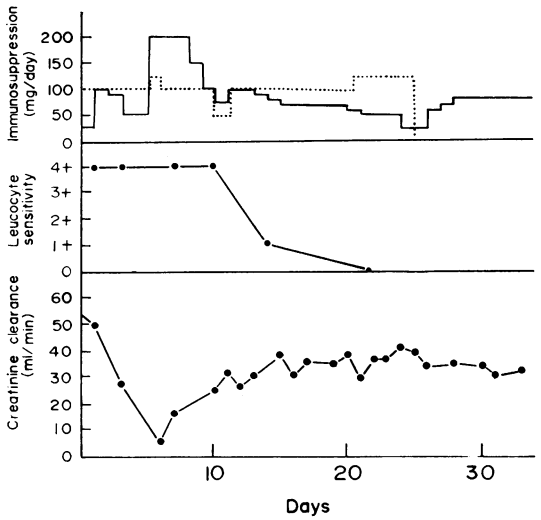

FIG. 2. Pattern of leucocyte migration inhibition occurring with graft rejection developing about the third day of post-transplantation. . . . . , Imuran; ——, Prednisone.

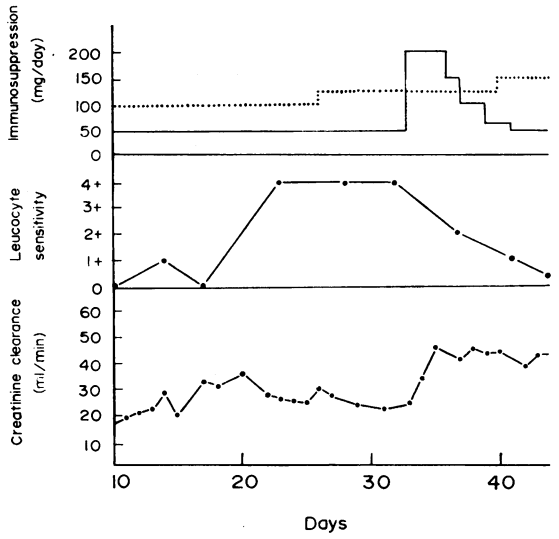

FIG. 3. Pattern of migration inhibition occurring about 10 days before clinical rejection was recognized. . . . . , Imuran; ——, Prednisone.

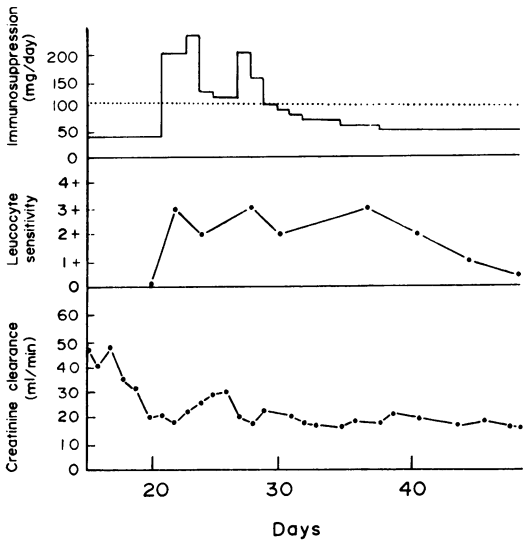

FIG. 4. Pattern of migration inhibition occurring after clinical rejection was recognized. ....., Imuran; ——, Prednisone.

Seven 'probable acute' rejection episodes were recognized. Three were preceded by migration inhibition, in one inhibition developed in the week following diagnosis of rejection and in three others it did not appear at all.

Thus if one takes all episodes definite and probable as being indicative of genuine acute graft rejection (fourteen cases) migration inhibition occurred in ten (72%) whilst four (28%) showed no evidence of sensitization.

In no case in which deterioration of renal function was initially attributed to acute rejection, but proved later to have been secondary to some other complication, was there evidence of migration inhibition.

In two patients going on to chronic rejection, migration inhibition developed.

There was only one instance in which migration inhibition occurred without recognized rejection. In retrospect, it is evident that in this patient the creatinine clearance which had been rising incrementally to the thirty-fourth day, plateaued at between 35 and 40 ml/min for about 12 days. The clinicians were apparently happy to accept this as a reasonable clearance; but on the forty-sixth day a further steady improvement began, and the final creatinine clearance reached on the seventh day was 65 ml/min. It seems quite likely that the migration inhibition test predicted a self-reversing mild rejection crisis between the thirty-fourth and forty-sixth days.

DISCUSSION

In this study we have followed patients sequentially with testing on a regular once or twice weekly basis in the belief that only by such constant evaluation of the test could its usefulness be assessed. We have shown that in a substantial proportion of cases the test can provide predictive evidence of impending graft rejection.

In as much as two patients have developed definite late migration inhibition in the face

of high dose steroid treatment, it seems unlikely that such therapy interferes substantially with the phenomenon of cell migration. The position with regard to Azathioprine is as yet not clear, since it is not our practice to make sudden large changes in Azathioprine dosage.

The specificity of macrophage migration inhibition has been amply demonstrated for both PPD in tuberculin +ve animals and for haptenprotein conjugates (David *et al.*, 1964). The test as modified by Söborg and Bendixen using peripheral blood leucocytes has been shown to be specific for Brucella antigen (Söborg & Bendixen, 1967) and for PPD (Rosenberg & David, 1970) although some workers have had difficulty in using the technique successfully (Kaltreider *et al.*, 1969). There is much about the method which is as yet not well understood. Nevertheless modifications to the technique by Rosenberg & David (1970) appear to make it a useful *in vitro* test for cellular hypersensitivity. In animal experiments this method has been shown to be consistent, immunologically specific, to correlate with the state of immunity (Falk, Collste & Moller, 1970) and to predict the onset of rejection of homografts (Eidemiller & Bell, 1972).

Whether or not there is immunological specificity for the reaction in terms of histocompatibility antigens is not clear. There is some evidence that migration inhibition may be acheived by the use of antigens other than those bearing the tissue type of the donor (Smith *et al.*, 1969; Galanaud *et al.*, 1972; Weeke, Weeke & Bendixen, 1970). An interesting variation of the technique used by Ellis, Read & Zabriskie (1971) makes use of the known immunological cross-reactivity between human renal GBM and streptococcal membrane antigens to demonstrate a correlation between cellular reactivity to streptococcal antigen and post-transplant renal function. We have used an extract of donor spleen cells as antigen in the belief that by doing so we were reproducing *in vitro* as closely as possible the immunological relationships that exist *in vivo*. The initial results are encouraging.

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