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Abstract

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**EARLY DIAGNOSIS OF ACUTE RENAL ALLOGRAFT
REJECTION: EFFICACY OF MACROPHAGE
MIGRATION INHIBITION TEST AS
AN IMMUNOLOGICAL DIAGNOSIS**

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Abstract. 1. Three cases of acute rejection were detected by macrophage migration inhibition tests (MIT) conducted directly on seven patients who had received renal allografts. The macrophage migration inhibitory factor (MIF) activity was positive in all cases 1-2 days before the appearance of acute rejection. 2. After the administration of a high dose of Solu-Medrol (1g/day for 3 days) to suppress the acute rejection, MIF activity recovered to its normal level 3 days later. These findings seem to indicate that MIT yields immunologically useful criteria for the early detection of an acute rejection.

Early detection of acute renal allograft rejection is an important problem for improving the functional survival of transplanted kidneys in our clinical practice. The chemical tests of blood and urine that are now used in clinics can detect the acute rejection reaction only when the disturbance of the transplanted kidney has advanced to a certain degree, hence it is urgently necessary to develop an immunologically early diagnostic method that is a true reflection of the rejection (1-4).

For the past several years, we have been studying diurnal changes of macrophage migration inhibitory factor (MIF) after renal allografting in rats (5) and dogs (6) by the macrophage migration inhibition test (MIT) developed by David (7) and Blom (8). As a results, it had become clear that MIF activity increases prior to the appearance of acute rejection. We report here our finding that this MIF also seems to be an effective immunologically early diagnostic method for detecting acute renal allograft rejection in man.

MATERIALS AND METHODS

Subjects

The subjects were seven cases of renal allograft managed in our department during the period from March, 1974 to September, 1975 (Table 1). MIT was conducted as a rule 2-3 times a week during the first postoperative month, twice a week from the beginning of the second month after operation until discharge

TABLE 1. CASES OF KIDNEY TRANSPLANTATION

No.	Age	Sex	Donor	HLA ^a match	MLC ^b	Rejec- tion	Creatinine clearance (ml/min.)	
LD-1	23	male	mother	3	9.8%	0	92	(1 y ^c . 6 m ^d .)
LD-2	28	female	„	2	8.8%	1	died	(10 m ^d .)
LD-3	23	male	father	2	8.6%	0	89	(6 m ^d .)
LD-4	26	female	mother	2	5.8%	1	28	(5 m ^d .)
LD-5	32	male	„	3	4.1%	0	95	(3 m ^d .)
LD-6	20	female	father	3	3.6%	1	85	(2 m ^d .)
LD-7	25	male	mother	2	4.1%	0	90	(1 m ^d .)

^a Human leucocyte antigen, ^b mixed lymphocyte culture, ^c year, ^d month

from hospital, and once a month after discharge. For prophylactic immunosuppression therapy, basic treatment consisted of the oral administration of Imuran and Medrol with 1g of Solu-Medrol being given i. v. on the first and second postoperative days. Acute rejection was observed in three (42.8%) of the seven cases once in each case. This was managed by pulse therapy of Solu-Medrol and regional irradiation of ⁶⁰Co (300R/day for 3 days).

Assay of Macrophage Migration Inhibition Factor (MIF)

MIF was measured by a modified George and Vaughan's method (9).

The preparation of donor specific antigen. Donor lymphocytes are isolated from 50ml of heparinized peripheral blood aspirated from a donor by the Ficoll-Conray method and adjusted to a concentration of 5×10^6 cells/ml. Four ml of medium TC-199 is added to one ml of such lymphocyte suspension, then ultrasonication performed for 3 min at 150 mA, 7 ϕ tip. The protein concentration is measured by spectrophotometry. Ampules are filled with 0.5 ml each of the sonicated suspension (usually 50 such ampules can be prepared), and these ampules are kept at -20°C . At the time of use, the suspension is diluted 10-fold and this serves as the antigen solution, having an average protein concentration of $190 \pm 36 \mu\text{g/ml}$.

Preparation of sensitized lymphocytes. Lymphocytes were isolated from 8 ml of heparinized peripheral blood by the Ficoll-Conray method and suspended in a concentration of 1×10^6 ml.

Preparation of guinea-pig macrophages. Fluid paraffin (20 ml) is injected into the peritoneal cavity of a guinea-pig, laparotomy performed 4 days later, and the abdominal cavity rinsed with Hanks' solution. The rinsed solution is centrifuged twice at 250g for 5 min each time, and the precipitate so obtained is placed in TC 199 solution, adjusted so that the number of cells contained is $2-5 \times 10^6$ cells/ml.

The macrophages and test lymphocytes are mixed in the ratio 10:1, and the mixed cell suspension is put under negative pressure into a hematocrit capillary tube with one end closed. The tube is centrifuged at 250g for 5 min, then cut at the boundary between the supernatant and the cell layer. The cell layer portion

is placed on a round cover-glass at the bottom of MacKness-type petri dish (1 ml), and fixed with silicon grease. One milliliter of antigen-containing medium is added, and this is then sealed with a cover-glass. As a control, a similar preparation is added to the medium not containing antigen. These are then incubated at 37°C for 24 hr. After culture, the migrations of cells around the opening of the capillary tube are measured with a projection microscope. Test cultures are done in triplicate. MIF activity is estimated after calculating % migration index by the following formula.

$$\text{MI (\%)} = \frac{\text{The average migration area with antigen}}{\text{The average migration area without antigen}} \times 100$$

A value of less than 80% is considered positive.

RESULTS

MIF Activity in the Non-acute Rejection Group (Fig. 1)

MIF activity (average value \pm S.D.%) throughout the entire posttransplantation course was $97.8 \pm 13.3\%$ in LD1, $99.8 \pm 2.5\%$ in LD3, $103.3 \pm 14.4\%$ in LD5, and $104.2 \pm 4.5\%$ in LD7, indicating that there was not a single case in which the MIF activity had become positive. The average MIF activity in these four cases was $101.2 \pm 17.2\%$, which we consider as within the normal range for MIF activity. MIF activity was near the border line of 80%, however, in 5/28 in LD1, 1/16 in LD3, 3/15 in LD5, and 1/10 in LD7. In such cases, the assay of MIF activity was repeated once more under careful observation of clinical symptoms and chemical data of blood and urine.

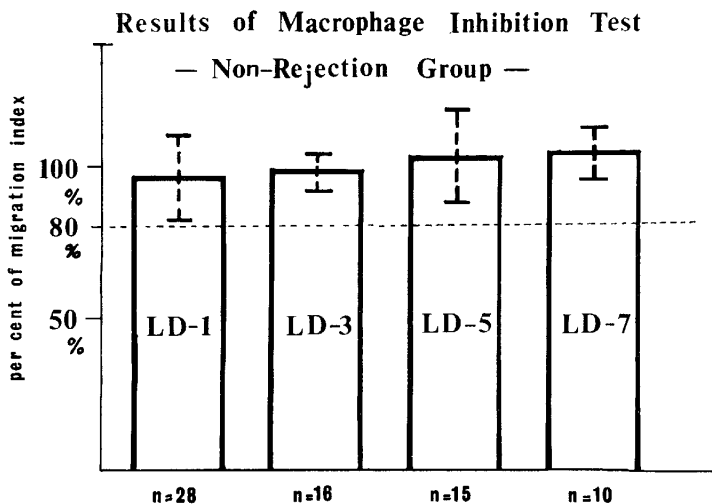


Fig. 1. Results of macrophage migration inhibition test (non-rejection group). In the non-rejection group, MIF activity was negative after renal allografting throughout the entire-clinical course.

Changes of MIF Activity in the Acute Rejection Group (Fig. 2)

Acute rejection was observed once in each of LD2, LD4 and LD6. The MIF activity prior to the rejection crisis in these three cases was $94.9 \pm 18.7\%$. A false positive result was obtained in three (13.5%) of twenty-two MI tests.

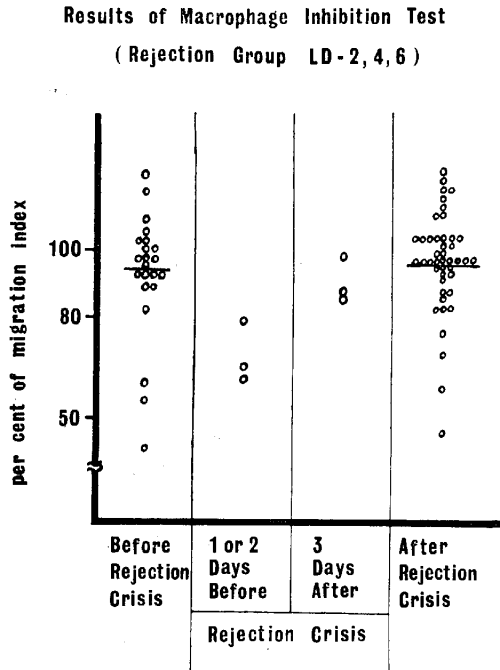


Fig. 2. Results of macrophage migration inhibition test (rejection group). In the acute rejection group, MIF turned positive 1-2 days before the rejection crisis and rapidly became negative 3 days after commencement of antirejection therapy.

If acute rejection is judged clinically by the rise in the BUN and serum creatinine (S-Cr) level, MIF activity 1-2 days before the rejection crisis without elevation of BUN or S-Cr was 60.4% in LD2 (BUN 26 mg/dl, S-Cr 1.2 mg/dl), 79% in LD4 (BUN 22 mg/dl, S-Cr 1 mg/dl), and 63.2% in LD6 (BUN 25 mg/dl, S-Cr 1.2 mg/dl), showing a definite elevation of cellular immune activity indicative of the impending rejection. Thereafter, these cases were diagnosed as acute rejection by the appearance of clinical symptoms and the elevation of BUN and S-Cr levels. When the dose of Medrol was increased and a high dose of 1 g/day Solu-Medrol was given i. v. for three days, MIF activity decreased rapidly to a normal range of 85% in LD2 (BUN 102 mg/dl, S-Cr 3.5 mg/dl), 86% in LD4 (BUN 106 mg/dl, S-Cr 7.2 mg/dl) and 86% in LD6 (BUN 65 mg/dl, S-Cr 3.2 mg/dl) 3 days after the commencement of such anti-rejection therapy, in spite of

persistent kidney insufficiency. Thereafter, MIF activity was $95.9 \pm 14.9\%$ and no longer showed any fluctuations in cases where the transplanted kidney functioned well three months after transplantation.

A brief explanation follows of the course of three typical cases that indicated acute rejection.

Case 1-LD2 (Fig. 3). A 28-year old female received her mother's kidney on Dec. 3, 1974. On the tenth post operative day (Medrol 48 mg/day, Imuran 150 mg/day) the body temperature suddenly rose to 38.1°C , while the white blood cell count was 8,600. Her renal function was normal with BUN 26 mg/dl and S-Cr 1.2 mg/dl, but MIF activity turned positive to 60.4%. On the twelfth day, BUN rose to 83 mg/dl and S-Cr to 2.8 mg/dl, indicating a rejection crisis. Hence pulse therapy with Solu-Medrol was started 1 g/day for 3 days, and hemodialysis performed twice. The renal function began to improve around the 23rd post-transplantation day.

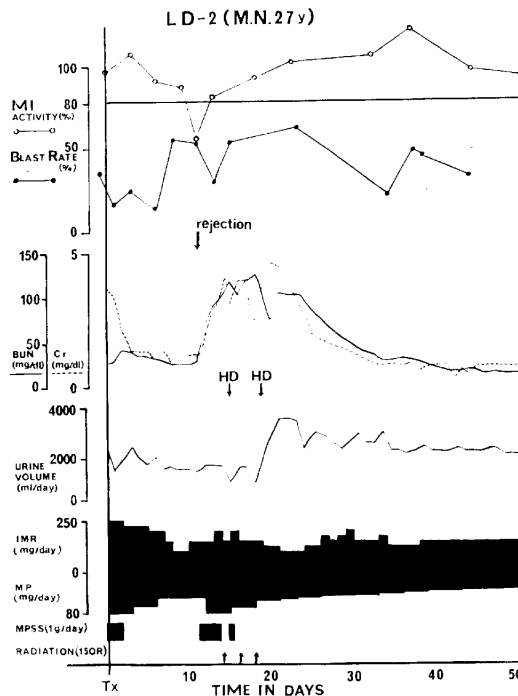


Fig. 3. Clinical course of LD-2. On the tenth transplantation day, body temperature suddenly rose to 38.1°C , renal function was normal, but MIF activity became positive at 60.4%. On the twelfth day, a rejection crisis was diagnosed clinically.

Case 2-LD4 (Fig. 4). A 26-year old female received her mother's kidney on May 20, 1975. Despite a favorable renal function of BUN 22 mg/dl and S-Cr 1.1 mg/dl without any clinical symptoms on the 16th day after transplantation (Medrol 48 mg/day, Imuran 150 mg/day) MIF activity became 79% which indicated a rise in the cellular immunity. Two days later, BUN was 49 mg/dl and S-Cr 2.7 mg/dl, a crisis was fully developed. In spite of the usual antirejection therapy, she required 8 hemodialysis over 4 weeks.

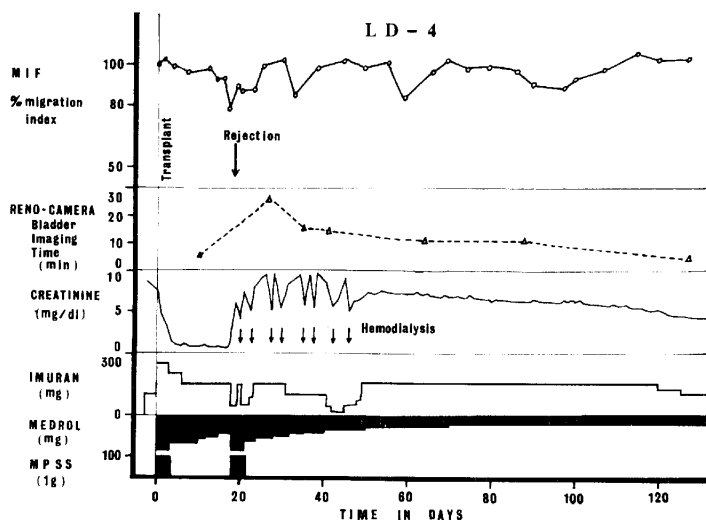


Fig. 4. Clinical course of LD-4. On the 16th day after transplantation, MIF turned positive to 79%. Two days later, a rejection crisis was confirmed.

Case 3-LD6 (Fig. 5). A 20-year old female received her father's kidney on August 5, 1975. Her postoperative clinical course progressed smoothly as shown by a BUN of 25 mg/dl and a S-Cr of 1.2 mg/dl on the 38th day (Medrol 28 mg/day and Imuran 125 mg/day). MIF activity, however, rose to 63.0%, so she was considered from our experience with two preceding cases to be in a state of impending rejection. Pulse therapy with Solu-Medrol was, therefore, commenced. On the following day, BUN rose to 35 mg/dl and S-Cr to 2.8 mg/dl, but her renal function had recovered one week later.

As described above, when MIF activity is taken as a criterion, it is suggested that MIF activity becomes positive before the rise in the concentration of BUN and S-Cr, and that the rejection can, as in these three cases, be predicted immunologically one or two days before clinical diagnosis of the rejection. However, it seems that the MIF activity does not parallel the severity of the acute rejection reaction as observed in these three cases.

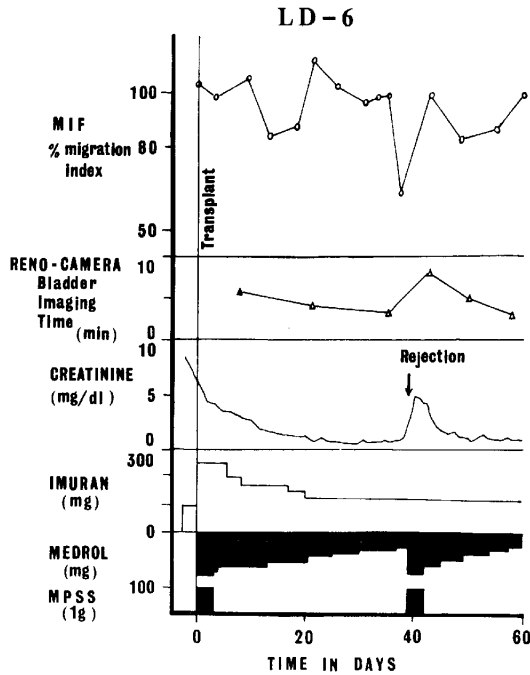


Fig. 5. Clinical course of LD-6. On the 38th transplantation day, MIF rose to 63.0% despite a good clinical course. On the following day, a rejection crisis was confirmed clinically.

DISCUSSION

When lymphocytes isolated from animals or man showing delayed hypersensitivity or cell mediated immunity are cultured with a specific corresponding antigen, a lymphokine, macrophage migration inhibitory factor (MIF), is liberated from the sensitized lymphocytes (10). This MIF inhibits the migration of normal guinea-pig macrophages from capillary tubes and induces a decrease in the radial migration area formed at the tip of a capillary tube, the so-called MI phenomenon (7, 8). In actual practice, the MIF activity is determined by the ratio of the migration areas (% migration index) of antigen-added, and non antigen-added groups.

There are two migration inhibition tests, *i. e.* the macrophage migration inhibition test (MIT) where guinea-pig macrophages are used as indicator cells (7, 8), and the leucocyte migration inhibition test (LIT) where polymorphonuclear leucocyte are used as the indicator cells, as developed by Sjöborg and Bendixen (11). There are no reports other than those of Turnipseed (12, 13) of MIT being used as a parameter of rejection. The LIT uses the buffy coat of blood from recipients, and the technique is so simple that most available reports are of LIT.

LIT has been employed as a parameter and its efficacy in acute renal allograft rejection reported by Smith *et al.* (14), Eva Weeks and Bendixen (15), Falk *et al.* (16), Dormont *et al.* (17), Wood *et al.* (18) and in liver transplantation by Eddleston *et al.* (19). However, Rocklin *et al.* report the difficulty of its reproducibility because polymorphonuclear leucocytes and sensitized lymphocytes cannot be maintained at a certain fixed ratio in number (20). By our MIT, it is always possible to maintain macrophages and sensitized lymphocytes in the ratio of 10 : 1 (David (7) states that 5% of sensitized lymphocytes would be sufficient) and it is clinically advantageous to execute MIT with as small an amount of blood as 5 ml.

The culture time required for diagnosis is generally 24 hr (7, 8), but it is said that liberation of MIF begins by the 6th hr of culture (21) and that the diagnosis is possible even at 8–12 hr. Generally crude or semisoluble antigen extracted from lymphocytes (12, 13, 16), spleen (16–18) or kidney (14, 17) is used as the specific antigen. The most important point to be borne in mind in the execution of MIT is the concentration of antigen to be added. If excessive antigen is added, a non-specific inhibition of the migration of macrophages occurs, possibly leading to misdiagnosis. There are reports in the range of 100 $\mu\text{g}/\text{ml}$ to 2 mg/ml of antigen concentration (12–19). When crude antigen of kidney extract and spleen extract was used, the optimal antigen concentration was 50–100 $\mu\text{g}/\text{ml}$ for Wood (18), 125 $\mu\text{g}/\text{ml}$ for Dormont *et al.* (17) and 100–200 $\mu\text{g}/\text{ml}$ for Weeke and Bendixen (13). Turnipseed *et al.* adjusted the number of donor lymphocytes to 25×10^6 cells/ml, subjected them to freeze-thawing seven times at -30°C , and used the suspension as the antigen containing solution, with an optimal concentration of 1–2 mg/ml (12, 13). We adjust the number of donor peripheral blood lymphocytes to 5×10^6 cells/ml, subject them to supersonication, and use at an optimal concentration of $190 \pm 36 \mu\text{g}/\text{ml}$.

When LIT was used in renal transplantation, the positive rate of migration inhibition during a rejection crisis was 100% for Wood *et al.* (18), 100% for Falk *et al.*, 95% for Dormont *et al.* (17), and 67% for Weeke (13). This was a marked correlation with the rejection crisis, but the positive rate in the prerejection period was 13.3% for Dormont *et al.* (17), and 16.7% for Falk *et al.* (16), a fairly low percentage. The sensitivity of LIT in the early diagnosis of acute rejection still appears to be a problem.

Direct MIT conducted periodically after renal allografting in rats, has demonstrated that MIF activity turns positive by the third day of transplantation, indicating that MIT is a useful method for early immunological diagnosis of acute rejection (5). Recently, the results of the clinical application of direct MIT were reported for the first time by Turnipseed and Cerilli (13). They performed MIT in 40 cases receiving kidneys from living donors, and in 16 (94.1%) out of 17 cases showing acute rejection, the MIF activity turned positive. Moreover,

30% of them converted to positive MIF activity about 10 days before a distinct clinical appearance of rejection, indicating that this method is excellent in monitoring cellular immunity after transplantation in both reproducibility and sensitivity. An interesting finding in their report was that an accelerated rejection occurred in two cases receiving renal allografts whose mixed lymphocyte culture (MLC) had been negative to prospective human leucocyte antigen (HLA) identical sibling donor before operation, but whose MIF was positive. The presensitization was detected by MIT before transplantation.

The next important problem will be the effect of immunosuppressive agents on MIF activity. Dormont *et al.* (17) state that Predonisolone under 0.5 mg/Kg/day and Imuran 2-3 mg/Kg/day as a maintenance dose hardly affect MIF activity, whereas MIF activity appears simultaneously with a rejection crisis, Turnipseed and Cerilli (13) have reported similar results. However, recently 20-30 mg/Kg/day of Solu-Medrol as pulse therapy is being recommended by many institutions, and it is said that the acute rejection can be suppressed in 90-95% of cases with renal allograft (22, 23). Similar to Dormont's report (17), MIF activity in our cases also turns negative within 1-3 days of the intravenous administration of Solu-Medrol in a high dose, irrespective of functional kidney recovery. It is not considered a suitable parameter for the follow up of acute rejection.

As described above, we consider that MIT does reflect well the cellular immune activation following transplantation and is a useful method for the diagnosis of impending rejection.

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