LEUCOCYTE MIGRATION AGAROSE TEST TO STUDY CELL-MEDIATED IMMUNITY IN AMOEBIASIS

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INTRODUCTION

The role of cell-mediated immune response (CMIR) in protective immunity in amoebiasis is still uncertain. Our previous study showed that lymphocytes from patients with amoebic liver abscess underwent blast transformation after exposure *in vitro* to the extract of *Entamoeba histolytica*, and the magnitude of the blastogenic response was not related to the number of precipitating bands in the immunoelectrophoresis test (Savanat *et al.*, 1973). Time course study showed that there was little or no blastogenic response in patients with early amoebic liver abscess to be followed by a heightened response some days later (Savanat *et al.*, 1973; Ortiz-Ortiz *et al.*, 1974). This apparent anergy in the early course of illness occurred without the associated reduction in T cell numbers (Landa *et al.*, 1976). Harris *et al.*, (1976) showed that blastogenic response was evident only in patients with amoebic liver abscess but not in patients with intestinal amoebiasis and symptomless carriers. Since most of the studies on CMIR in amoebiasis were done using the blast transformation test, its real correlation with CMIR has been questioned (Savanat *et al.*, 1973). The objective of the present study was to appraise CMIR in patients with amoebiasis using the leucocyte migration agarose test in comparison with the humoral immune response (HIR) using the indirect haemagglutination (IHA) tests and to measure quantitatively the magnitude of CMIR and HIR in these patients at varying period of time after the onset of clinical illness.

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MATERIALS AND METHODS

Thirty-five patients with amoebic liver abscess admitted to various hospitals in Bangkok were studied (30 patients from Siriraj Hospital, 2 patients from the Hospital for Tropical Diseases, one patient each from Phra Mongkut Klao Hospital, Bamrajnaradun Hospital and Phayathai Hospital). The diagnosis was based on clinical manifestations with sterile anchovy sauce pus of the liver aspirate and favourable therapeutic response to anti-amoebic drugs. Among these patients, only ten were available for the follow-up study. The patients were arbitrarily divided into 5 groups according to the onset of clinical illness and the time of testing (Table 1).

Twenty-two healthy individuals working in the Faculty of Tropical Medicine, Mahidol University served as controls.

Twenty ml of venous blood were drawn, 15 ml of which were mixed with preservative free heparin (Evans Medical, Liverpool) so that the final concentration of heparin was 20 units/ml of blood to be used for preparation of leucocytes for CMIR testing. The remaining 5 ml of blood was allowed to clot, the serum separated for serological testing and stored at -20°C until used.

The antigen used was the extract of *E. histolytica* strain HK-9 originally obtained from Dr. L. S. Diamond (NIH, Bethesda)

and prepared according to the technique described previously (Savanat *et al.*, 1973).

Leucocyte migration agarose test (LMAT) originally described by Bendixen *et al.*, (1976) was used with slight modification. The heparinised blood was mixed with 3.5% dextran T 250 (Pharmacia Fine Chemical, Uppsala, Sweden) in the ratio of 2 : 1 and left stand upright at 37°C for 45 to 60 minutes to allow the red cells to sediment. The leucocyte rich plasma was centrifuged at 1000 g at 4°C for 5 minutes and the remaining red blood cell lysed by addition of 1 ml of 0.83% NH_4Cl solution for 3 minutes, resuspended in RPMI 1640 without serum, and washed 3 times by centrifugation. After final washing, the cell number was adjusted to contain 2.1 × 10^8 per ml in RPMI 1640 containing 10% AB serum. Forty µl of the leucocyte suspension was mixed with 10 µl of the antigen solution containing 100 and 200 mg protein/ml. The cell antigen mixture was incubated in a water bath at 37°C for 30 minutes, and 10 µl portion of the cell suspension was placed in quadruplicate by means of 10 µl micropipette into 3 mm diameter wells in the 60 × 15 mm tissue culture dish (Falcon 3002) containing 1% agarose (Sigma type III, high EEO No. A-6138) in RPMI 1640 (Gibco) plus 10% AB serum and 100 µg gentamicin per ml. The final antigen concentration in each well was 200 and 400 µg. The agarose plates were incubated at 37°C in a humidified candle jar for 20-24 hours. Thereafter, the preparation was examined under an inverted microscope (Unitron, series N) to be ascertained that the leucocytes had migrated at a satisfactory distance. The preparation was then fixed with the mixture of glacial acetic acid-picric acid solution (saturated picric acid 100 ml + glacial acetic acid 20 ml), followed by careful manual removal of the agarose gel, leaving areas of cell migration permanently preserved (Fig.1)

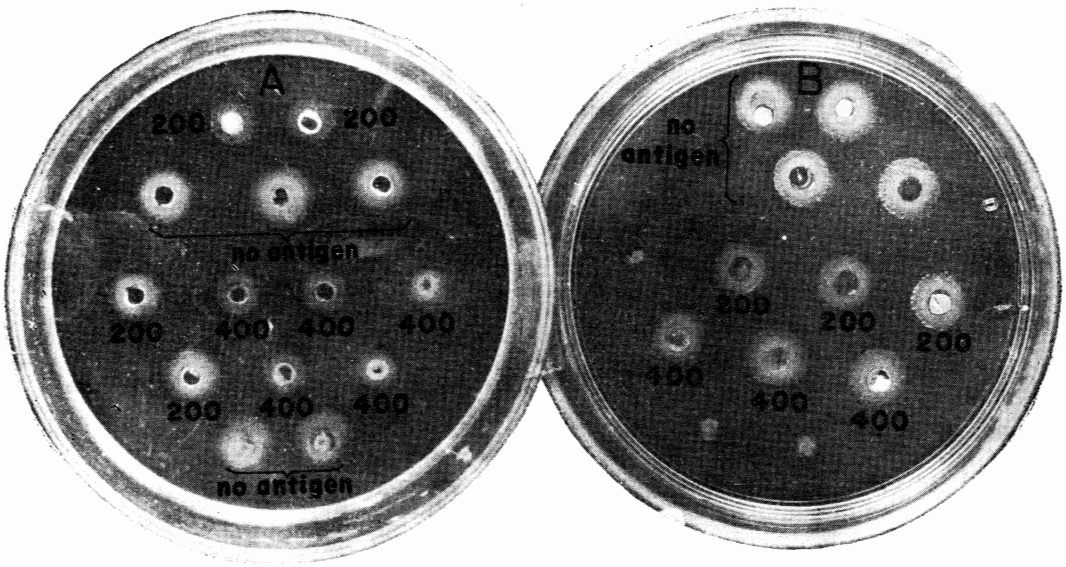

Fig. 1 — Leucocyte migration agarose test (LMAT) showing inhibition of migration in the absence (control) and in the presence of 200 and 400 µg of antigen.
A. Patients with amoebic liver abscess.
B. Healthy control.

The plate was then projected onto a screen. at the base of the Unitron inverted microscope and the area of cell migration recorded by tracing on a weighing paper with uniform weight (Eli Lilly and Co.). The paper was weighed on an analytical balance (Mettler H-20 T), and the weight of the central well was substracted to yield only the actual weight of the area of cell migration. The migration index (M.I.) was determined according to the formula :-

$$\text{M.I.} = \frac{\text{area (weight) of cell migration in the presence of antigen}}{\text{area (weight) of cell migration in the absence of antigen}}$$

Indirect haemagglutination test (IHA) using stabilized sensitized cells was developed in our laboratory (Chongsanguan *et al.*, pers. comm.). Sheep red blood cells (SRBC) were sensitized with the *E. histolytica* antigen according to the technique of Milgram *et al.*, (1966). The sensitized SRBC was stabilized by pyruvic aldehyde (Pfaltz and Bauer, Stamford, Connecticut, U.S.A.) according to the technique of Hambie *et al.*, (1977). The stabilized sensitized cells were lyophilized in the presence of 12.5% solution of tween 80 according to the technique of Meuwissen and Leewenberg (1972) and stored at-10°C until used. Preliminary test showed that their activities were not changed significantly after 4 months storage. Prior to use, the lyophilised sensitized SRBC were reconstituted with distilled water and the suspension was subjected to ultrasonic treatment for approximately 45 seconds using an ultrasonic disintegrator (MSE) at an amplitude of 8 microns.

Sensitized SRBC, 0.025 ml was added to 0.075 ml serial fourfold dilutions of the serum with the initial dilution of 1:20 and 1:40 so that the conventional twofold dilutions of the serum was obtained. The plate was kept at room temperature for 1 hour and then at 4°C overnight. As controls, a suspension of non-sensitized SRBC similarly treated was added to the lowest serum dilution. If positive, the serum was absorbed with washed SRBC prior to testing according to the technique of Meuwissen and Leeuwenberg (1972) in order to remove the Forssman antibody.

RESULTS

Cell-mediated immune response

Results of CMIR in patients with amoebiasis and in the controls are shown in Table 1

Table 1

Humoral and cell-mediated immune responses in patients with amoebiasis at various time after the onset of clinical symptoms.

Time(days) after onset of clinical symptoms	No. tested	IHA		Leucocyte migration agarose test			
		Mean log titer±S.E.	No. pos. titer 1:640	200 µg antigen		400 µg antigen	
				Mean M.I. ± S.E.	No. pos. M.I.<0.825	Mean M.I. ± S.E.	No pos. M.I.<0.712
≤10	4	3.94±0.26	4 (100)	0.88±0.05	1 (25)	0.77±0.07	1 (25)
11-30	17	3.62±0.11	17 (100)	0.67±. 06	12 (70)	0.49±0.06	15 (88)
31-60	7	3.58±0.16	7 (100)	0.59±0.09	6 (86)	0.16±0.08	7 (100)
61-90	3	3.31±0.20	3 (100)	0.78±0.05	2 (66)	0.30±0.18	3 (100)
≥90	4	3.56±0.40	4 (100)	0.83±0.07	2 (50)	0.17±0.09	4 (100)
Total	35	3.62±0.47	35 (100)	0.71±0.23	23 (65.7)	0.40±0.30	30 (85.7)

Percentage positive shown in parenthesis.

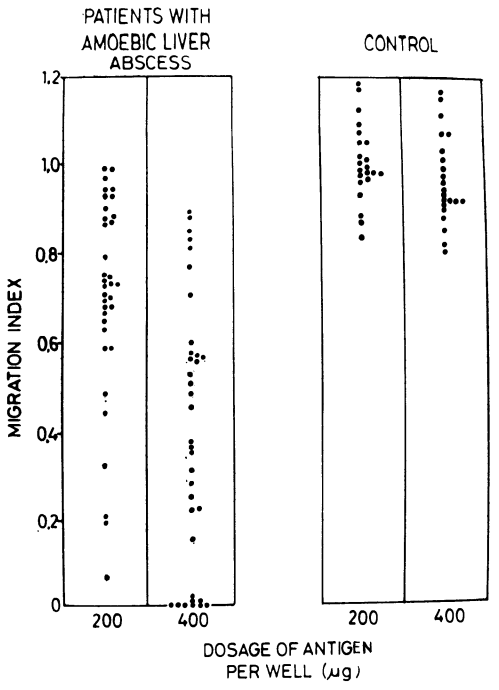

Fig. 2—Migration index in patients with amoebic liver abscess and controls in the presence of 200 and 400 µg of the antigen of *E. histolytica*.

and Fig. 2. The mean M.I. ± S.E. from patients with amoebiasis were 0.71 ± 0.23 and 0.40 ± 0.30 in the presence of 200 and 400 µg antigen respectively. Such M.I. are significantly different from those of the controls (M.I. ± S.E.=1.01 ± 0.09 and 0.97 ± 0.10 in the presence of 200 and 400 µg antigen respectively). The lower 95% confidence limits of M.I. in the controls in the presence of 200 and 400 µg antigen were 0.83 and 0.77 respectively, and these figures were accordingly chosen as a threshold M.I. for distinguishing positive from negative CMIR at these 2 dosages. Among 35 patients tested, the number positive in the presence of 200 µg and 400 µg antigens were 23 (65.7%) and 30 (85.7%) respectively (Table 1). The relationship between CMIR and time after the onset of clinical illness is shown in Table 2 and Fig. 3. It was evident that M.I. was high during the first 10 days of illness followed thereafter by lower M.I. with the lowest value during 31-60 days of illness. It was found also that after 60 days, there was a rise in M.I. when 200 µg antigen was used, but little further change was observed with that of 400 µg antigen. Analysis of variance showed that there was no significant difference among these 5 groups when 200 µg antigen was used ($\rho > 0.05$). In the presence of 400 µg antigen, the difference between the 5 groups was highly significant ($\rho < 0.01$). When results of the LMAT in patients using 2 dosages of antigen were compared and analysed by dependent "t" test, the mean M.I. at 400 µg dosage was significantly lower than those when 200 µg was used ($\rho < 0.01$).

Table 2

Cell-mediated immunity in patients with amoebiasis and in the controls in the presence of 200 and 400 µg of antigen.

Antigen dose	Migration index			
	Patients with amoebiasis		Controls	
	Mean ± S.E.	range	Mean ± S.E.	range
200 µg	0.71 ± 0.23	0.07-0.99	1.01 ± 0.09	0.85-1.19
400 µg	0.40 ± 0.30	0-0.89	0.97 ± 0.10	0.81-1.16

Humoral immune response

The geometric mean of the indirect haemagglutinating titers of 35 sera from patients

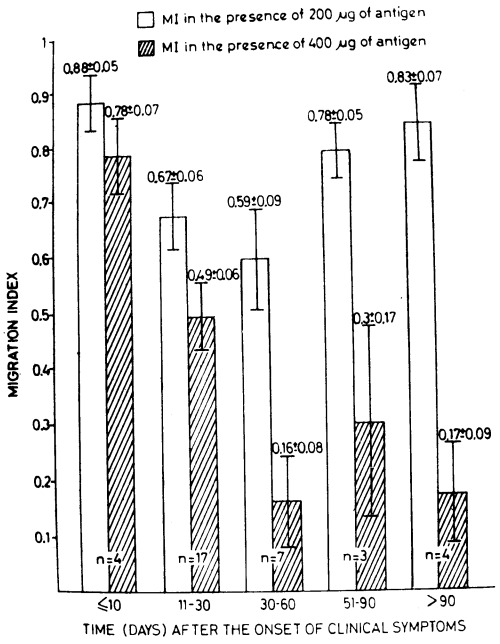

Fig. 3—Migration index (mean ± S.E.) in the presence of 200 and 400 µg of antigen in patients with amoebic liver abscess at various time after the onset of clinical illness

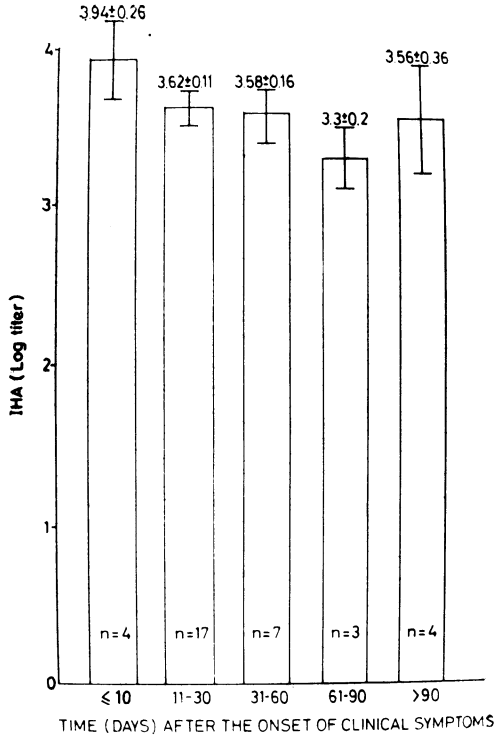

Fig. 4—Mean log IHA titer (mean ± S.E.) in patients with amoebic liver abscess at various time after the onset of clinical illness.

Table 3

Frequency distribution of IHA titer in patients with amoebiasis and in controls.

Titer	Controls	Patients
≤ 1:40	6	-
1:80	2	-
1:160	1	-
1:320	8	-
1:640	5	1
1:1280	-	6
1:2560	-	14
1:5120	-	4
1:10240	-	5
1:20480	-	2
≥ 1:40960	-	3

with amoebiasis was 1 : 5120 with a range from 1 : 640 to > 1 : 40960, whereas that of the control was 1 : 80 with a range from 1 : 40-1 : 640. The frequency distribution in each IHA titer is shown in Table 3. Time course analysis (Table 1, Fig. 4) showed that the IHA titer was high in the sera taken within 10 days after the onset of clinical symptoms, followed by a slight decrease in titer thereafter. Analysis of variance showed that the difference in mean titers among these 5 groups was significant ($0.01 < \rho < 0.05$).

The relationship between IHA test and LMAT were assessed by the correlation and regression analysis. It was found that there was no correlation between the IHA titers and M.I. neither at the antigen dosage of 200 µg ($r = 0.15$, $\rho > 0.05$) nor 400 µg ($r = 0.05$, $\rho > 0.05$).

DISCUSSION

In the usual test for migration inhibitory factor, there was no permanent record of the area of cell migration in the migration chamber or Petri dish for reassessment if required. The LMAT modified from that of Bendixen *et al.*, (1976) made such record possible. The use of candle jar in place of CO_2 incubator renders possible the test for cell-mediated immunity to be done even in a poorly equipped clinical laboratory by cutting cost of capital investment on an expensive CO_2 incubator and the cost of the CO_2 supply.

With LMAT technique it was clearly shown that cell-mediated immunity was developed in patients with amoebic liver abscess and thus confirmed the results previously reported using the blast transformation test (Savanat *et al.*, 1973; Harris and Bray, 1976) and the MIF test (Ortiz-Ortiz *et al.*, 1975). The LMAT positivity was related to the time after the clinical onset of the disease and the antigen dosage. In the first 10 days of illness, only 1 of 4 patients was positive, whereas later in the course of illness, the percentage positivity was markedly raised. This apparent anergy has been documented also in patients before treatment followed by positive cell-mediated immune response after recovery as measured by the MIF test (Ortiz-Ortiz *et al.*, 1975) and skin testing (Landa *et al.*, 1976). The diminished cell-mediated reaction was specific for amoebiasis since skin reaction to other non-related antigen was not altered, and the rosette test for T cells was normal (Landa *et al.*, 1976). The higher dosage (400 μg) of the antigen gave significantly lower migration index than the lower dosage (200 μg) ($\rho < 0.01$). The 400 μg dosage should be selected for use in the future study because of its higher sensitivity. It was also optimum for the blast transformation test (Savanat *et al.*, 1973). Obviously this dosage was higher than that (100 μg/ml) used in the MIF test in the capillary tube by Ortiz-Ortiz *et al.*, (1975) and in the leucocyte migration test by Petchclai *et al.* (1980).

Analysis of the relationship between the result of LMAT and the time after the onset of clinical symptoms showed persistence of reaction when 400 μg antigen was used. In contrast, the response to 200 μg antigen was high during 31-60 days of illness followed thereafter by a diminution of the reaction. There is no known explanation for this phenomenon, but it could be possible that specific recognition of the antigen by T cell was waning with time, and could be triggered only by a strong stimulus of higher antigen dose.

To determine when after the onset of clinical symptom that the LMAT became positive and how long could it persist after therapy, our results showed that more than 70% of the patients tested were positive between 11-30 days after the onset of clinical illness, and could thus be interpreted that in most cases the cell-mediated immunity had developed during this period. The immunity may disappear as early as 8 months since LMAT in one patient which could be followed up to this period was negative.

By the IHA test, it was found that significantly higher antibody was demonstrated in patients with amoebiasis. Time course study showed that the titer was high even on the first 10 days of illness and slightly but significantly declined thereafter. Analysis of he relationship between IHA antibody titers and cell-mediated immune response showed no correlation. This is not surprising since the two types of immune responses are dependent on two distinct populations of lymphocytes. This finding substantiates our previous observation that lymphocyte transformation in the presence of the amoebic antigen did not correlate with the antibody

response detected by the IEP test (Savanat *et al.*, 1973).

SUMMARY

The leucocyte migration agarose test (LMAT) was used to measure quantitatively the magnitude of cell-mediated immunity (CMI) in 35 patients with amoebic liver abscess and 22 healthy controls. LMAT was positive in 65.7% and 85.7% of patients with amoebiasis in the presence of 200 μg and 400 μg of the amoeba extract respectively, whereas the test in all 22 healthy controls was negative. Time course studies showed that within 10 days after the onset of clinical illness, only 1 of 4 patients was positive. Thereafter the percentage positivity was raised, especially when 400 μg antigen was used. Maximum CMI response was apparent between 31-60 days after the onset of clinical illness.

The indirect haemagglutination (IHA) test showed that all patients and 5 of 22 healthy controls were positive. There was no correlation between IHA titers and the magnitude of LMAT reaction.

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