Cellular immunity in systemic lupus erythematosus as evidenced *in vitro* by leucocyte migration inhibition test

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SUMMARY A leucocyte migration inhibition test was performed on 26 patients with systemic lupus erythematosus (SLE) and on 35 control subjects using three different antigens, fetal calf thymus DNA, baker's yeast RNA, and calf thymus extractable nuclear antigen (ENA). Leucocyte migration was inhibited by DNA in 17 out of 26 SLE patients (65.3%), and in only 2 of the 35 controls (5.7%). When RNA or ENA was added none of the patients or controls showed inhibition. In SLE patients migration inhibition by DNA was significantly correlated with the presence of proteinuria and/or granular casts in urinary sediment. When the migration inhibition test was positive, immunofluorescence verified active histology of the glomeruli obtained by a percutaneous renal biopsy.

Humoral immunity in the pathogenesis of systemic lupus erythematosus (SLE) has been well documented, but the role of cell-mediated immunity has yet to be thoroughly investigated. Although it is still a matter for debate, several workers (Bitter *et al.*, 1971; Federlin and Helmke, 1972; Horwitz, 1972; Messner *et al.*, 1973; Williams *et al.*, 1973) have found depressed cellular immunity in active SLE. Goldman *et al.* (1972), studying patients with lupus nephritis, reported a stimulatory effect by native DNA in lymphocytes cultured *in vitro*, and Abe *et al.* (1973) observed the macrophage migration inhibition factor to DNA *in vitro* in their study of patients with SLE and the nephrotic syndrome.

To further evaluate the possible role of delayed hypersensitivity to DNA in the pathogenesis of SLE nephritis, leucocyte migration inhibition tests were performed on our patients using calf thymus DNA as antigen. The results were analysed together with the immunohistological study of kidney biopsy specimens obtained simultaneously.

Patients and methods

Twenty-six SLE cases (Table 1) were diagnosed according to the ARA criteria (Cohen *et al.*, 1971). All had 'definite' SLE, exhibiting four or more manifestations plus the presence of a positive LE preparation and/or anti-DNA antibody. Kidney biopsies were performed on 22 patients. 23 normal subjects and 22 patients with other diseases served as the controls (Table 2).

COMPLEMENT (CH₅₀)

Levels were determined according to the method described previously using 50% haemolysis as the end-point (Lange *et al.*, 1960).

DNA

DNA used for the haemagglutination test was prepared by the method of Kay *et al.* (1952), using quick-frozen calf thymus. The procedure was stopped at step 7. The amount of protein measured using the method of Lowry *et al.* (1951) was found to be insignificant.

A haemagglutination test for DNA antibody was performed using a modified version of the method of Sharp *et al.* (1971). Tanned human type O erythrocytes were used for agglutination instead of sheep red blood cells (R. J. Winchester, personal communication, 1974). LE cell preparations were

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Table 1 Migration inhibition index in patients with SLE

Case no.	Age (yrs)	Sex	Duration of SLE (yrs)	Outcome	Migration inhibition index			
					PPD 20µg/ml (%)	DNA 200µg/ml (%)	RNA 500µg/ml (%)	ENA 2400µg/ml (%)
1	46	F	5	Alive	70	74, 70, 99, 95	87	123
2	19	F	5	"	63, 132	60, 95, 115, 94	103	
3	42	F	9	"		61, 77		
4	20	F	6	"		67, 64		90
5	35	F	5	"		73, 69		
6	36	F	3	"		21, 59, 67, 80, 91	85	
7	17	M	10	"		64, 88	99	
8	20	M	9 m	"		61, 75, 107		
9	16	F	3	"	89	55, 125		
10	23	F	8	"	55	77, 67, 35, 111, 78		
11	31	F	2	"		97, 61		
12	80	F		Died		55		
13	18	F	1	Alive		68, 100, 68		
14	31	M	2	"		91, 91, 76, 69	97	
15	30	F	1	"		80, 72		
16	36	F		Died		95, 69		94
17	40	F	2	Alive		68, 104		97, 85
18	27	F	3	"	106	83, 90, 80		
19	26	F	1	"		78, 78		
20	25	F	11	"		93, 100		
21	20	F		"		98, 95		
22	30	F	11	"		81		
23	31	M	4	"	81	100, 113	86	
24	35	F	3	"		102, 97	87	
25	21	F	9	"		86, 95		
26	54	F	4	"		113		

ENA=extractable nuclear antigen.

made by the 2-hour blood clot technique (Hargraves *et al.*, 1948).

LEUCOCYTE MIGRATION INHIBITION TEST (MIT)

Tuberculin PPD

Materials without preservatives were purchased from Parke-Davis (Detroit, Mich.). 20 µg/ml was in the culture medium.

DNA

Commercially available fetal calf thymus DNA was obtained from Worthington Chemicals (Freehold, N.J.). Over 90% of the lyophilized material was composed of double-stranded DNA. 200 µg/ml DNA was used in the culture medium.

RNA

Baker's yeast RNA was purchased from Worthington Chemicals and was used in a concentration of 500 µg/ml.

Extractable nuclear antigen (ENA)

Prepared according to the method of Sharp *et al.* (1972). A concentration of 2400 µg/ml was used.

The MIT was performed according to the method of Søborg and Bendixen (1967) with some modifications. 20 ml peripheral blood was drawn into a disposable plastic syringe containing 2000 units heparin (Lipohedin, Riker, Northridge, Calif.), held

upright for 60 to 90 minutes in a 37°C incubator to obtain leucocyte-rich plasma (buffy coat). The plasma plus the same volume of normal saline were mixed in a sterilized 15 ml test tube, centrifuged at 400 g for 10 minutes, then the supernatant was discarded by aspiration. After adding Ringer's lactate, the sediment was washed twice by centrifugation at 400 g for 5 minutes. The WBC count, differential count, and Trypan-blue exclusion test for viability were performed. The cell pellet obtained was resuspended in a Ringer's lactate solution having a concentration of 20×10^6 mononuclear cells/ml.

The cell suspension was put into capillary tubes (inner diameter 1.1–1.2 mm), one end sealed with clay, then centrifuged at 400 g for 5 minutes. The cell column which formed at the bottom was cut at the supernatant-cell interphase, and put into a McKanes plastic chamber (Univers Mekaniska Verkstad AB Herrhagsv, 98 Endkede, Sweden), affixing the closed end to side-wall of the chamber with stop-cock grease. The chamber was filled with TC Medium 199 (Difco, Detroit, Mich.) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 20% fetal calf serum (heat-inactivated, Flow Lab, Rockville, M.D.). pH was preadjusted to 7.4 with 8% NaHCO₃.

Triplicate cultures were set up for each antigen and additional triplicate chambers containing the same volume of Ringer's lactate as that of the

Table 2 Migration inhibition index in normal controls and in patients with other diseases

Case no.	Age (yrs)	Sex	Clinical diagnosis	Migration inhibition index			
				PPD 20µg/ml (%)	DNA 200µg/ml (%)	RNA 500µg/ml (%)	ENA 2400µg/ml (%)
Normal controls							
27	30	M	Healthy		84, 95		187
28	37	M	"		74, 83		101
29	36	M	"		117		
30	37	M	"	73	85, 83		
31	31	M	"	84, 108	79		
32	23	F	"	105	92	108	
33	38	M	"		77	105	
34	45	M	"		106	105	
35	24	F	"	108	91	101	
36	33	M	"		79	97	
37	25	M	"		106	129	
38	37	M	"		110	74	
39	34	F	"		90	100	
40	28	F	"	107	60, 84, 86	122	
41	42	M	"		104	137	
42	30	F	"		108		
43	28	F	"		86		
44	26	F	"		93		
45	20	F	"		114		
46	30	M	"			107	
47	30	F	"	63			
48	28	M	"	94			
49	31	F	"	94			
Patients with other diseases							
50	22	F	Acute-poststreptococcal glomerulonephritis;		110		
51	19	F	Rheumatoid arthritis		101		
52	24	F	Orthostatic proteinuria		108		
53	30	F	Rheumatoid arthritis		120		
54	18	F	Rheumatic heart disease		85	74	
55	20	M	Nephrotic syndrome		93	103	
56	25	F	Arthritis		99		
57	40	M	Diabetes mellitus		84	95	
58	43	F	Obesity		103	95	
59	31	M	Anaemia		90		
60	17	F	Factor 7 deficiency anaemia		94		
61	27	F	Chronic active hepatitis		95		
62	21	M	Nephrotic syndrome		100, 92		
63	25	M	Nephrotic syndrome		124		
64	43	F	Chronic active hepatitis		101		
65	45	F	Sarcoidosis		90		
66	50	M	Tuberculosis	43			
67	42	M	"	44			
68	27	M	"	58, 68			
69	61	M	"	50			
70	25	M	"	40			
71	22	M	Hereditary nephritis	82			

antigen served as the control. Each chamber was put in a 37°C CO₂-incubator for 21 hours. A phot enlarger was used to draw the migratory area on paper. The image was cut out, weighed, and the migration inhibition index (MII) calculated as

$$\frac{\text{mg image of chamber with antigen}}{\text{mg image of chamber without antigen}} \times 100 (\%).$$

Considering that the mean -1 standard deviation of the MII with DNA was found to be 92% - 15.5 (76.5%) for 19 normal controls (Table 2, Fig. 2), an MII of <75% was arbitrarily chosen to represent

inhibition. Sterile technique was kept throughout the culture procedure.

PREPARATION AND EXAMINATION OF KIDNEY TISSUE

Kidney biopsy specimens for light microscopy were fixed in 10% neutral formalin and embedded in paraffin. 2-3 µm thick sections were stained with a haematoxylin-eosin, periodic acid schiff (PAS), and periodic acid silver methenamine.

Specimens for immunohistology were prepared by immediately embedding the biopsy cylinder in fresh rat liver and freezing for 3 minutes in an

isopentane-dry ice mixture (Lange *et al.*, 1966). 3 μ m thick sections were sliced on a -20°C cryostat, air dried, stained with FITC-labelled sera (Sober *et al.*, 1956; Riggs *et al.*, 1960) for 30 minutes in a moist chamber, and washed in 0.01 M phosphate-buffered saline pH 7.1. They were examined under a Leitz ultraviolet microscope with a 200 W Osram B high tension mercury vapour bulb and photographed with a Leitz Orthomat camera using Anscochrome 200 outdoor film.

Specimens for electron microscopy were fixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in epon. Ultrathin sections were prepared on a Reichert II microtome. Sections were stained with uranyl acetate and lead citrate, then examined under a Hitachi HU-11A electron microscope.

Results

PRELIMINARY STUDIES

In order to verify that MIT can be a good parameter of cellular immunity, a skin test for tuberculin PPD was simultaneously performed on 13 subjects (Fig. 1). The mean ± 1 SD of the MII ($53\% \pm 12.1$) for the subjects with positive skin tests was found to be

Fig. 1 Migration inhibition test with tuberculin PPD as antigen and skin test with the same antigen.

significantly lower than that ($87\% \pm 5.9$) of subjects with negative skin tests ($P < 0.001$). The mean ± 1 SD ($51\% \pm 10.7$) for the tuberculous cases was significantly different from that of the SLE patients ($85\% \pm 26.7$) and 9 normal subjects ($93\% \pm 16.5$) ($P < 0.02$ and $P < 0.001$ respectively).

The MIT with DNA antigen was performed on 26 SLE patients, 17 of them (65.3%) showing inhibition at some time during the course of their illness, with a mean (± 1 SD) MII* of $71\% \pm 19.7$ (Table 1, Fig. 2). In the 35 controls, comprised of 19 normal subjects and 16 patients with other diseases (Table 2, Fig. 2), no migration inhibition was observed except in 2 normal subjects (Cases 26 and 40, Table 2) who showed inhibition with MII titres of 74% and 60%, respectively. The mean of the MII titre in the SLE subjects was significantly

Fig. 2 Migration inhibition test with DNA as antigen in patients with SLE and in control subjects.

*Each subject, either patient or control, was represented by the minimal MII titre when MIT was done two or more times.

lower than that in the control subjects as a whole ($96\% \pm 14.1$) ($P<0.001$). The difference in mean values between the SLE patients ($71\% \pm 19.7$) and the normal controls ($92\% \pm 15.5$) or that between SLE patients and patients with other diseases ($99\% \pm 11.5$) was also significant ($P<0.001$ in both cases; Fig. 2, Tables 1, 2), while the difference in mean values between normals and patients with other diseases was nonsignificant ($P<0.1$; Fig. 2, Table 2). Of the 2 normal subjects whose MIT showed inhibition, the one with the borderline test (Case 28, 74%, Table 2) was found to have a normal MII (83%) at the second estimation. The other with an MII titre of 60% (Case 40, Table 2) was a female doctor with a past history of unexplained leucopenia and a family history of a lymphoproliferative disorder.

The results of the MIT with DNA as antigen in SLE patients did not correlate significantly with fever, erythema, arthralgia, and/or Raynaud's phenomenon, or values of ESR, WBC, Coombs's test, LE cells, anti-DNA antibody, or complement ($P>0.05$). However, the mean MII in symptomatic patients with positive laboratory tests tended to be higher than the mean of the asymptomatic patients. A few individual cases showed good correlation between the MII titre and the presence of signs,

symptoms, and positive laboratory tests. One representative case follows.

Case 8 (Fig. 3, Table 1)

A 20-year-old Chinese male student, admitted with a fever of 37.8°C , facial erythema, and arthralgia. Clinical data showed Coombs's test 4+, leucopenia, raised ESR, low complement, and proteinuria 3+. Histological examination of his kidney under light microscope showed proliferative glomerulonephritis. MII on admission was 61%. Prednisone 100 mg once daily was started and during the following month on steroids clinical and laboratory data quickly returned to normal as did MII titres.

MIT AND RENAL INVOLVEMENT IN SLE

The mean MII of SLE patients with proteinuria* and/or granular casts ($70.3\% \pm 18.2$) was significantly lower than that of patients with normal urinalysis ($89.3\% \pm 15.7$) ($P<0.01$, Fig. 4). For those patients whose MIT and urinalysis were repeated two or more times, only the highest and lowest MII titres were considered in order to minimize sampling error.

Kidney biopsy was performed on 22 SLE patients, and in 16 it was done within a week of MII esti-

*Trace proteinuria was not taken as significant proteinuria and was therefore grouped together with no proteinuria.

Fig. 3 Case 8. Migration inhibition test with DNA as antigen and clinical course of a patient with SLE.

Fig. 4 Migration inhibition test with DNA as antigen and proteinuria and/or granular casts in patients with SLE.

mations (Table 3). All 6 MIT-positive patients showed fluorescent staining by immunohistology. In 5 staining was moderate to strong, and in the remaining patient weak. In the 10 MIT-negative patients fluorescence was negative in 1, weak in strong in 3, and the remaining case was not studied by this method.

EFFECT OF STEROID THERAPY ON THE MIT
No overall correlation was observed between MII titres and duration or dosage of steroids. The MII of one patient (Case 14, Table 1) who followed downhill course clinically was negative, but became positive despite a high dose of prednisone. In 2 other cases (Cases 8, 17, Table 1), whose clinical courses were smooth, the MII became negative after massive steroid therapy.

The results of the MIT with RNA as antigen were analysed in 7 SLE patients and 14 control subjects (10 normals and 4 patients with other diseases), as shown in Tables 1 and 2. Although all the tests were essentially negative with the mean MII being $92\% \pm 7.4$ for SLE, $108\% \pm 17.9$ for normals, and $92\% \pm 12.4$ for patients with other diseases, respectively, the difference in means between SLE and normal was significant ($P < 0.05$). The results of the MIT with ENA in 5 SLE patients and 3 normal controls were all negative (Tables 1, 2).

Discussion

Since Søborg and Bendixen (1967) first established the MIT as the simplified modification of the

Table 3 Migration inhibition index (MII) and renal histology in patients with SLE

Case no.	MII/DNA 200µg/ml (%)	Renal histology				
		Light microscopy	Immunofluorescence	Electron microscopy		
				Subendothelial deposit	Intra-mesangial deposit	Virus-like particles
MIT-positive patients						
3	61	Diffuse proliferative	2+	—	+	—
4	67		1+			
6	21	Proliferative	3+			
9	55	Mild increase in mesangium	2+	—	—	—
11	61	Mild proliferative + mesangial	3+	—	—	—
17	68	Proliferative	3+			
MIT-negative patients						
7	88		1+			
8	107	Proliferative				
10	77	Proliferative	1+			
13	100	Minimal increase in mesangium	1+			
14	91	Focal proliferative	3+	—	+	—
18	90	Proliferative	1+			
20	100		3+			
21	98	Subendothelial	1+	—	—	—
24	102	Mesangial	3+	—	—	+
26	113	Proliferative	—	—	—	—

macrophage migration inhibition test, this assay system has been successfully used by many using particulate antigens (Søborg, 1967). When the soluble antigens were in use their validity was doubted (Lockshin, 1969; Kaltreider *et al.*, 1969). Kaltreider *et al.* (1969) tried to find the correlation between the MIT and the results of skin tests using tuberculin PPD as the antigen. Later, Rosenberg and David (1970) re-evaluated and confirmed the validity of the MIT with PPD, ascribing prior failures to technical problems. We also reconfirmed this point using the same model. MIT correlated well with the skin test, using tuberculin PPD as antigen (Fig. 1, Tables 1, 2).

The involvement of humoral-type immunity in SLE has been extensively studied and its pathogenetic role well documented, e.g. detection of antinuclear antibodies and/or free DNA in patients' sera (Seligmann, 1957; Deicher *et al.*, 1959; Holman *et al.*, 1959; Stollar *et al.*, 1962; Tan *et al.*, 1966; Pincus *et al.*, 1969; Hughes, 1971; Harbeck *et al.*, 1973), a lowered level of serum complement (Lange *et al.*, 1960; Lagrue *et al.*, 1966; Asano *et al.*, 1968), and the demonstration of DNA-anti-DNA antibody complex together with complement in glomeruli (McCluskey *et al.*, 1966; Lange *et al.*, 1966; Koffler *et al.*, 1967), etc. On the other hand, fewer studies have been done on cell-mediated immunity and its role in the disease process is still controversial.

With regard to the hypersensitivity to DNA, since our report (Ores and Lange, 1964) on positive skin tests to DNA, Patrucco *et al.* (1967) published their results on a blast-like transformation of lymphocytes cultured *in vitro* with calf thymus DNA. A similar report was published independently by one of us (Okubo, 1968). Podleski and Podleski (1973) observed *in vitro* specific cytotoxic effects of lymphocytes from SLE against DNA-coated human cells. Recently, Abe *et al.* (1973) reported that the migration inhibition factor was produced by incubating lymphocytes taken from SLE patients with calf thymus DNA. Similar positive results were reported by Galanaud *et al.* (1971), Dormont *et al.* (1972), Moulias *et al.* (1972), using a Bendixen-type direct MIT. Our study, using the same direct MIT, gave positive results in 65.3% of SLE patients, using double-stranded calf thymus DNA as antigen (Fig. 2, Table 1). In the control subjects the MII was negative except in 2 cases (Cases 28, 40). In agreement with the observations of Moulias *et al.* (1972) and Dormont *et al.* (1972), no correlation was found between the MIT and other clinical or laboratory data of SLE excepting nephropathy (see below).

However, other workers reported negative results using similar methods, e.g. Reinert *et al.* (1971) (lymphocyte transformation test), Horwitz (1972)

(skin test and lymphocyte culture *in vitro*), Dormont *et al.* (1972) (lymphocyte transformation test, although results by MIT were positive), Hahn *et al.* (1973a, b) (skin test and lymphocyte culture *in vitro*), Federlin and Helmke (1972) (migration inhibition test).

In order to understand the discrepancies many factors have to be considered, such as differences in the techniques used, consequently, the different aspects studied (skin test, lymphoblastogenesis, or migration inhibition factor), the effect of steroid or other immunosuppressive therapy, and most importantly the presence of disturbed or deficient cellular immunity in SLE (Bitter *et al.*, 1971; Horwitz, 1972; Federlin and Helmke, 1972; Williams *et al.*, 1973; Messner *et al.*, 1973). Steroid therapy, in agreement with previous reports, did not seem to affect profoundly the lymphocytic reactivity *in vitro*. In one patient (Case 14) the migration inhibition became apparent only after initiation of steroid therapy, while in 2 other patients (Cases 8, 17) no further inhibition was observed during steroid administration. Our result seemed related more to the disease activity itself than to dosage or duration of steroid therapy.

That disturbed cell-mediated immunity occurs in SLE has long been suggested by frequent false-negative skin tests with tuberculin antigen. Bitter *et al.* (1971) and Horwitz (1972) observed a depressed intradermal reaction to prevalent bacterial or fungal antigens in their SLE patients. As for the stimulation study by phytohaemagglutinin, Bitter *et al.* (1971) could not produce a stimulation response, while Horwitz (1972) found a normal response. Using the MIT, Federlin and Helmke (1972) showed depressed cellular hypersensitivity to DNA in SLE, while Williams *et al.* (1973) and Messner *et al.* (1973) reported a decrease in the number of T lymphocytes in the peripheral blood of SLE patients. In one of our cases (Case 2) the MII with PPD as antigen fluctuated from 63 to 132%, possibly suggesting that deranged cellular immunity may affect the MII titre.

Recently it has been suggested that cell-mediated immunity may play a role in the development of lupus nephropathy (Goldman *et al.*, 1972; Abe *et al.*, 1973). Goldman *et al.* (1972) reported that 3 of 6 SLE patients with active nephritis showed a positive response to rabbit native DNA when lymphocytes were cultured *in vitro*. All 10 SLE patients with the nephrotic syndrome studied by Abe *et al.* (1973) showed a positive migration inhibition factor sometime during the clinical course. Abe *et al.* further referred to the possible role of cell-mediated immunity in the perpetuation of renal disease.

Our results were compatible with previous data in that the MIT was positively correlated with the

presence of proteinuria and/or granular casts in the urinary sediment. We performed kidney biopsies in 22 of our 26 SLE patients, aiming for prompt diagnosis and treatment of their renal disease. In 16 of these 22 patients the MIT was performed simultaneously (Table 3). MIT-positive patients showed active immunohistology as seen by positive staining with FITC-labelled antihuman IgG and antihuman β_2 globulin (2+ to 3+ staining in 5 of 6 MIT-positive patients, Table 3). Also in 3 out of 10 MIT-negative patients (Cases 14, 20, 24, Table 3), 3+ staining was observed. This may have something to do with derangement of cell-mediated immunity inherent in SLE, and perhaps some patients with very active lupus nephropathy have no more T lymphocytes left in their circulation responsible for the positive MIT. Further investigation, including studies of T and B lymphocytes circulating in peripheral blood of SLE patients with active immunohistology at the time of the MIT study, may be necessary to clarify the role of cellular immunity in the renal disease of SLE.

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