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The *in vitro* effect of thymic humoral factor and levamisole on peripheral blood lymphocytes in systemic lupus erythematosus patients

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SUMMARY

The *in vitro* effect of thymic humoral factor (THF) and levamisole on E rosette-forming cells in the peripheral blood of seventeen patients with systemic lupus erythematosus (SLE) was studied.

Patients with active disease showed a low number of E rosette-forming cells. A significant rise in the number of E rosettes was obtained after incubation with both THF and levamisole. No such effect was observed on lymphocytes from patients with inactive disease and normal controls.

In seven patients, three with active disease and four with well-controlled disease, short-term cultures were performed. The effect of THF on E rosettes was found to be the same before and after the short-term cultures.

Possible mechanisms, by which THF (on the one hand) and levamisole (on the other) may increase the number of E rosettes *in vitro*, are discussed.

INTRODUCTION

Evidence that T-cell function is defective in patients with active SLE has been accumulated (Rosenthal & Franklin, 1975; Scheinberg & Cathcart, 1974; Michlmayr *et al.*, 1976).

A decrease in E rosette-forming cells in acutely ill SLE patients has been demonstrated by Messner, Lindstrom & Williams (1973), Scheinberg & Cathcart (1974); Scheinberg, Cathcart & Goldstein (1975) and also in our laboratory (Michalevicz, Many & Ramot, 1977). However, a definitive explanation for this phenomenon has not yet been suggested.

A humoral factor obtained from the thymus has been shown to participate in lymphoid cell proliferation and maturation, namely that thymic hormones transform immature T cells into mature or immunocompetent cells (Trainin, 1974; Trainin *et al.*, 1974; Kook & Trainin, 1974; Bach *et al.*, 1975; White, 1975). The administration of thymosin to NZB mice restored T-cell deficiencies to normal but did not prevent the development of autoimmune diseases (Talal *et al.*, 1975). It has recently been shown that thymosin increased the number of E rosettes and decreased the number of 'null' cells in the peripheral blood of SLE patients (Scheinberg *et al.*, 1975).

At approximately the same time, levamisole, an antihelminthic drug, was shown to increase the number of E rosettes, *in vitro* and *in vivo*, in patients with Hodgkin's disease (Ramot *et al.*, 1976). This immunomodulating drug has also been administered to patients with rheumatoid arthritis and to two SLE patients (Huskisson *et al.*, 1976; Gordon & Keenan, 1975). These authors reported that levamisole therapy restored the immunological parameters of T-cell function back to normal.

The observations on the influence of both thymic hormones and levamisole on lymphocytes prompted us to examine and compare their *in vitro* effect on the lymphocytes of our SLE patients..

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MATERIALS AND METHODS

Seventeen patients with SLE were examined, eleven with active disease and six with well-controlled SLE. All patients were females and their ages ranged between 20 and 40 years. Twenty laboratory personnel, matched for age and sex, were tested as controls. All patients in the group satisfied the criteria of the American Rheumatism Association and their condition was considered as either 'active' or 'inactive', as previously described (Michalevicz *et al.*, 1977). Eight patients with active disease were without treatment at the time of the study.

Thymic humoral factor (THF) was prepared by one of us (N. Trainin) (Kook, Yakir & Trainin, 1975). The isolation of THF consists of homogenization of calf thymus, and the ultracentrifugation and dialysis of the material. The active dialysate, used in this work, was adjusted to a standard concentration of 1mg protein/ml. The active principle is a polypeptide of about 3000 mol. wt.

The effect of THF on E rosettes was evaluated in fifteen SLE patients; that of levamisole on twelve patients. In ten of them the effect of THF and levamisole were studied simultaneously.

30 ml of blood were obtained in heparin and the mononuclear cells were separated on a Ficoll–Metrizoate gradient, according to the method of Böyum (1968). The cell suspension contained approximately 90% lymphocytes. It was incubated for 0·5 hr at 37°C. The number of T cells was determined by their ability to form E rosettes, as described by Wybran, Chantter & Fudenberg (1973). In brief, 0·25 ml of a lymphocyte suspension containing 10^6 cells was mixed with 0·25 ml of a fresh 1% sheep red cell suspension and 50 μl of calf serum. After centrifugation for 2 min at 200 *g*, the mixture was incubated at 18°C for 1 hr. After gentle mixing, the E rosette-forming cells were counted by means of a phase microscope.

The B cells were detected by the method of Taylor *et al.* (1971), using the direct immunofluorescent technique. For demonstration of membrane immunoglobulins, 10^6 cells were incubated for 30 min at 0°C with FITC-conjugated goat antisera to human IgG, IgA and IgM (Meloy). After three washings with PBS, the cells were examined under a Zeiss fluoromicroscope. 200 mononuclear cells were examined in oil immersion fields and the percentage of the fluorescent lymphocytes was calculated. While enumerating the B cells, the monocytes were excluded morphologically by the phase-contrast microscope. This was possible after finding a good correlation between the phase-contrast microscopy and α-naphtylacetate esterase and peroxidase staining, as well as EA rosettes, according to Shevah *et al.*, (1972).

The effect of THF was studied by incubating $3–5 \times 10^6$ lymphocytes per ml PBS, with 150 μg THF (found to be the optimal dose in this system) for 30 min at 37°C. As controls, either PBS or a 1% bovine albumin solution was used, instead of THF. The cells were then washed twice and tested for the two previously described markers.

The same technique was used with 50 μg per ml of levamisole which was incubated with the lymphocytes for 30 min at 37°C. E rosettes were determined before and after the incubation of lymphocytes with levamisole. Viability of lymphocytes before and after incubation with THF and with levamisole was determined by trypan blue dye exclusion.

The effect of THF was also studied on lymphocytes exposed to short-term culture. This was performed in seven SLE patients, three with active disease and four with well-controlled disease. 10^6 lymphocytes per ml in Eagle's medium with 10% AB serum were placed in T25 flasks, gassed, and incubated at 37°C overnight in 5% CO_2 and 95% air.

Only cultures in which lymphocyte recovery and viability were higher than 95% were used. Cells were washed twice before use. Recovered lymphocytes from each patient were divided into two aliquots: those treated by THF or not. The number of E rosettes were then determined and compared.

RESULTS

Patients with SLE are lymphopenic both in the active stage of the disease and, to a lesser degree, in the inactive stage (Table 1).

The *in vitro* effect of THF on E rosette-forming and Ig-bearing cells in patients with SLE (active and inactive disease) is shown in Table 1. THF raised the number of E rosette-forming cells in patients with active disease from $35·4 \pm 7·73$ (mean ± s.d.) to $56·9 \pm 9·41$%. The effect was found to be highly significant ($P < 0·0005$ by the Student's *t*-test). No such effect was found on the percentage of Ig-bearing cells: $33·1 \pm 5·74$ *vs* $30·0 \pm 4·4$ ($P > 0·15$). In patients with inactive disease, THF induced no effect on E rosettes: $60·2 \pm 8·7$ *vs* $58·5 \pm 13·0$ ($P > 0·45$); nor on Ig-bearing cells: $23·7 \pm 7·4$ *vs* $17·7 \pm 4·84$ ($P > 0·15$). No effect was observed on lymphocytes of normals: $62 \pm 8·7$ *vs* $60·4 \pm 10·8$.

No increase in the number of E rosettes occurred when buffer alone or bovine albumin was added (data not shown). The number of Ig-bearing cells was not changed significantly by THF in the two groups of SLE patients.

The effect of levamisole on E rosettes in the peripheral blood of patients with active and inactive disease is shown in Table 2. Levamisole raised the number of E rosettes in patients with active disease from $33·3 \pm 5·5$ (mean ± s.d.) to $53·3 \pm 4·5$% ($P < 0·0005$). On the other hand, levamisole had no effect on the E rosette-forming cells in patients with inactive disease: $60·5 \pm 9·4$ *vs* $53·0 \pm 11·2$ ($P > 0·2$).

TABLE 1. Effect of THF on E rosette-forming and Ig-bearing cells in normal controls and in patients with active and inactive SLE

Patients	Lympho-cytes (per mm³)	E rosettes				Ig-bearing cells			
		Without THF		With THF		Without THF		With THF	
		(%)	Absolute number (per mm³)	(%)	Absolute number (per mm³)	(%)	Absolute number (per mm³)	(%)	Absolute number (per mm³)
Active SLE									
D.E.	574	30	172	52	299	32	184	28	161
K.O.	600	30	180	55	330	45	270	34	204
L.E.	880	27	238	50	440	38	334	36	317
S.O.	504	28	141	50	252	28	142	—	—
K.S.	640	42	268	60	384	28	179	22	141
R.S.	600	35	210	52	312	30	180	30	180
R.W.	760	34	258	45	342	—	—	—	—
W.S.	900	40	360	60	540	—	—	—	—
A.S.	660	30	198	55	363	45	297		
S.S.	880	52	457	77	678	—	—	—	224
K.R.	720	42	302	70	504	32	230	30	216
Mean±s.d.	702±137	35·4±7·7	253·3±92·5	56·9±9·4	403·9±126	33·1±5·7	227±67·6	30±4·4	206±57·3
Inactive SLE									
E.G.	640	52	333	46	294	26	166	25	160
N.M.	1200	66	792	60	720	16	192	15	180
A.S.	800	52	416	52	416	33	264	16	128
N.L.	1280	71	908	78	998	20	256	15	192
Mean±s.d.	980±309	60·2±8·7	612·4±281	58·5±13	607±316·3	23·7±7·4	219·6±24	17·7±4·8	165±28
Normals (n=20)									
Mean±s.d.	2350±645	62·0±8·7	1404±125·4	60·4±10·8	—	24±3·8	434±64·6	—	—

TABLE 2. Effect of levamisole on E rosette-forming cells in SLE patients and normal controls

Patients	E rosettes (%)	
	Without levamisole	With levamisole
Active SLE		
D.E.	30	50
K.O.	30	52
L.E.	27	50
S.O.	28	48
K.S.	42	60
R.S.	35	52
R.W.	34	60
W.S.	40	55
Mean±s.d.	33·3±5·5	53·3±4·5
Inactive SLE		
E.G.	52	43
N.M.	66	69
M.M.	53	48
A.H.	71	52
Mean±s.d.	60·5±9·4	53·0±11·2
Normals (n = 20)		
Mean±s.d.	62·4±12·5	54·6±10·8

TABLE 3. The *in vitro* effect of THF on E rosettes in SLE patients before and after short-term cultures

	E rosettes (%)			
	0 hr		24 hr	
Patients	Without THF	With THF	Without THF	With THF
Active SLE				
S.O.	28	50	32	40
S.S.	52	77	60	68
K.R.	42	70	50	72
Inactive SLE				
E.G.	52	46	50	n.d.
N.M.	66	60	60	56
N.L.	71	78	80	80
K.M.*	72	70	76	74

* Patient not included in Table 1.

Again this effect did not occur in normal controls: $62{\cdot}4 \pm 12{\cdot}5$ *vs* $54{\cdot}6 \pm 10{\cdot}0$. The number of E rosettes was not changed significantly after short-term cultures. Furthermore, the same effect of THF on E rosette-forming cells was seen before and after the cultures. The results are shown in Table 3.

In some patients, repeated examinations were performed at different times and proved to be consistent.

DISCUSSION

The low number of E rosette-forming cells in patients with active SLE found in our study has been described by several authors. Winchester *et al.* (1974) have suggested that this low number of E rosettes may be due to the presence of cold anti-lymphocyte antibodies. Our experiments, in which the cells were incubated at 37°C for 0·5 hr, washed and then tested for E rosettes, seem to rule out this possibility. Furthermore, no significant increase in the number of E-rosettes was observed after short-term culture of the lymphocytes.

On the other hand, *in vitro* incubation of lymphocytes from active SLE, with either THF or levamisole, significantly increased the number of E rosettes. A number of explanations could be proposed for this observation. The low number of E rosettes may be due to the presence, in the peripheral blood, of an immature subpopulation of T cells, as suggested by Scheinberg *et al.* (1975). Maturation of incompetent T cells through regulation of the intracellular cyclic AMP by THF, as suggested by Kook & Trainin (1974), or by levamisole, as described by Lima *et al.* (1974), would explain the increase in the number of E rosettes. The levels of intracellular cyclic AMP in lymphocytes of SLE patients, before and after treatment with THF or levamisole, have, however, not been determined as yet.

Another possibility, not mutually exclusive, is that a blocking substance present on the surface of a subpopulation of T cells is responsible for the low number of E rosettes, as found in Hodgkin's disease. The *in vitro* incubation of Hodgkin's disease peripheral blood lymphocytes with levamisole significantly increased the number of E rosettes (Ramot *et al.*, 1976). Moroz *et al.* (1976) have shown that the blocking substance which levamisole removes from these lymphocytes is ferritin. The fact that both levamisole and THF increased E rosette-formation in active SLE patients could be explained by the removal of a blocking substance from the surface of the T lymphocytes by either. Whether this substance is ferritin or some other glycoprotein remains to be determined.

We believe that further characterization of the T-cell subpopulation affected by levamisole and THF, as well as a better understanding of their mechanism of action in SLE, is needed before these drugs are clinically applied.

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REFERENCES

BACH, J.F., DARDENNE, M., PLEAU, J.M. & BACH, M.A. (1975) International conference on thymus factors in immunity. *Ann. N.Y. Acad. Sci.* **249**, 186.

BÖYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by centrifugation and sedimentation at 1 *g*. *Scand. J. clin. Lab. Invest.* **21**, Suppl. 97, 77.

GORDON, B.L. & KEENAN, J.P. (1975) The treatment of systemic lupus erythematosus (SLE) with T-cell immunostimulant drug levamisole: a case report. *Ann. Allergy*, **35**, 343.

HUSKISSON, E.C., DIEPPE, P.A., SCOTT, J., TRAPNELL, J., BALME, N.W. & WILLOUGHBY, D.A. (1976) Immunostimulant therapy with levamisole for rheumatoid arthritis. *Lancet*, **i**, 393.

KOOK, A.I. & TRAININ, N. (1974) Hormone-like activity of a thymus humoral factor on the induction of immune competence in lymphoid cells. *J. exp. Med.* **139**, 193.

KOOK, A.I., YAKIR, Y. & TRAININ, N. (1975) Isolation and partial chemical characterization of THF, a thymus hormone involved in immune maturation of lymphoid cells. *Cell. Immunol.* **19**, 151.

LIMA, A.O., JAVIERRE, M.Q., DA SILVA, W.D. & CAMARA, D.S. (1974) Immunological phagocytosis: effects of drug on phosphodiesterase activity. *Experimentia*, **30**, 945.

MESSNER, R.P., LINDSTROM, F.D. & WILLIAMS, R.C. (1973) Peripheral blood lymphocyte cell surface markers during the course of systemic lupus erythematosus. *J. clin. Invest.* **52**, 3046.

MICHALEVICZ, R., MANY, A. & RAMOT, B. (1977) Peripheral blood lymphocytes in systemic lupus erythematosus—Relation to activity. *Acta Haemat.* **57**, 199.

MICHLMAYR, G., PATHOULI, C., HUBER, C. & HUBER, M. (1976) Antibodies for T lymphocytes in systemic lupus erythematosus. *Clin. exp. Immunol.* **24**, 18.

MOROZ, CH., LAHAT, N., BINIAMINOV, M. & RAMOT, B. (1977) Ferritin on the surface of lymphocytes of Hodgkin's disease patients—a possible blocking substance removed by levamisole. *Clin. exp. Immunol.* **29**, 30.

RAMOT, B., BINIAMINOV, M., SHOHAM, CH. & ROSENTHAL, E. (1976) Effect of levamisole on E rosette forming cells *in vivo* and *in vitro* in Hodgkin's disease. *New Engl. J. Med.* **294**, 809.

ROSENTHAL, C.J. & FRANKLIN, E.C. (1975) Depression of cellular mediated immunity in systemic lupus erythematosus. Relation to disease activity. *Arthr. and Rheum.* **18**, 207.

SCHEINBERG, M.A. & CATHCART, E.S. (1974) B and T cell lymphopenia in systemic lupus erythematosus. *Cell. Immunol.* **12**, 309.

SCHEINBERG, M.A., CATHCART, E.S. & GOLDSTEIN, A.L. (1975) Thymosin-induced reduction of 'null cells' in peripheral blood lymphocytes of patients with systemic lupus erythematosus. *Lancet*, **i**, 424.

SHEVACH, E.M., HEBERMAN, R., FRANK, M.M. & GREAN, I. (1972) Receptors for complement and immunoglobulin on human leukemic cells and human lymphoblastoid cell lines. *J. clin. Invest.* **51**, 1933.

TALAL, N., DAUPHINEE, M.J., PILLARISETTY, R. & GOLDBLUM, R. (1975) Effect of thymosin on thymocyte proliferation and autoimmunity in NZB mice. *Ann. N.Y. Acad. Sci.* **249**, 438.

TAYLOR, D.B., DUFFUS, W.P.H., RAFF, M.O. & DE PETRIS, S. (1971) Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature: New Biol.* **233**, 225.

TRAININ, N. (1974) Thymic hormones and the immune response. *Physiol. Rev.* **54**, 272.

TRAININ, N., SMALL, M., ZIPORI, D., UMIEL, T., KOOK, I.A. & ROTTER, V. (1974) *The Biological Activity of Thymic Hormones* (ed. D.W. van Bekkum), p. 117. Kooyber Scientific Publications, Rotterdam.

WHITE, A. & GOLDSTEIN, A.L. (1975) The endocrine role of the thymus and its hormone, thymosin, in the regulation of the growth and maturation of host immunological competence. *Adv. Metab. Discord.* **8**, 359.

WINCHESTER, R.J., WINFIELD, J.B., SIEGAL, F., WERNET, P., BENTWICH, Z. & KUNKEL, H.G. (1974) Analysis of lymphocytes from patients with arthritis and systemic lupus erythematosus. *J. clin. Invest.* **54**, 1082.

WYBRAN, J., CHANTTER, S. & FUDENBERG, H.H. (1973) Isolation of normal T cells in chronic lymphatic leukaemia. *Lancet*, **i**, 126.