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AN *IN VITRO* ASSAY OF
LEUKOCYTE MIGRATION INHIBITORY ACTIVITY
FROM HUMAN LYMPHOCYTES
STIMULATED WITH CONCAVALIN A

By

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When lymphocytes from animals or humans with cell-mediated hypersensitivity are exposed *in vitro* to the specific antigen, they generate and/or release a group of soluble factors commonly known as "lymphokines" (9).

Several investigators have reported that lymphocytes incubated with the plant mitogens phytohaemagglutinin or concanavalin A (Con-A) produce factors with similar biological activities. Thus, skin reactive factor (12, 19), lymphotoxin (10) and guinea pig macrophage migration inhibitory factor (MIF) (12, 20) are produced by mitogen-stimulated lymphocytes. Con-A and specific antigen both induce the production of migration inhibitory factors which are identical, as characterized by disc electrophoresis, isopycnic centrifugation in CsCl solutions, and susceptibility to the enzymes chymotrypsin and neuraminidase. However, the elution patterns of antigen-released and mitogen-released MIF on Sephadex G-100 are similar but not identical; this suggests a heterogeneity, which may be due to minor differences in charged groups of the molecule (13). By the same techniques, Rocklin et al. 1972 (18) have demonstrated obvious differences between human and guinea pig antigen-released MIF; these differences con-

cern molecular weight, electrophoretic mobility, density and neuraminidase susceptibility.

Since lymphokines are assumed to play an important role as mediators of cellular immunity, their isolation and further chemical characterization is of great interest. The small amount of lymphocyte-released material and the presence of serum and antigen or mitogen in the test systems seriously hamper attempts to isolate these factors. Furthermore, the reported species differences in the structure of MIF seem to necessitate further studies in the human system.

The purpose of the present study was to examine whether a migration inhibitory effect on human peripheral leukocytes (LMIA) could be demonstrated in serum- and mitogen-free supernatants of Con-A stimulated human lymphocytes. The technique utilizes the fact that Con-A can be removed from culture supernatants by binding to cross-linked dextran gels (1). The method presumably may prove valuable for generating larger amounts of lymphokines for purification and identification work. Furthermore, the potentials of the technique as an assay for unspecific LMIA production and/or release capacity of peripheral blood lymphocytes in various clinical conditions seem promising and are under investigation.

MATERIAL AND METHODS

Subjects

The study comprised 20 persons. Patients with diseases suspected of influencing cellular immunity, and patients under treatment with corticosteroids or cytostatics were not included, with a few exceptions, as pointed out.

Lymphocyte Culture

Venous blood was collected in 10 ml polysterole tubes (Nuncclon tubes®, Nunc, Roskilde, Denmark) containing 250 i. u. heparin (heparin with 5000 i. u. per ml, Leo, Copenhagen, Denmark) and 3 ml Hanks' balanced salt solution. Mononuclear cells were separated by centrifuging for 40 min at 220 g in an isopaqueficoll gradient (Lymphoprep®, Nyegaard & Co, Oslo, Norway). A 95-99 per cent pure mononuclear cell population was obtained which contained only a few per cent monocytes. The cells collected in a 10 ml polysterole tube were then washed three times in Hanks' balanced salt solution and suspended in

serum-free medium TC-199 with penicillin 67 i.u. per ml, and streptomycin 67 μ g per ml (Difco Laboratories, Michigan, U.S.A.) (TC-199).

Lymphokine Production

250 μ g concanavalin A (Pharmacia, Uppsala, Sweden) in 50 μ l TC-199 was added to 3 ml cell suspension (2.5×10^6 cells per ml), and incubated at 37° C in 2 per cent CO₂ in atmospheric air saturated with water vapour. After 22 h, the culture was terminated by centrifuging for 10 min at 670 g, and 50 μ l TC-199 was added to the cell-free supernatant.

In each test, a parallel Con-A-reconstituted control supernatant was made in the following way: A 3 ml cell suspension, identical with the sample, was added to 50 μ l TC-199 without Con-A and incubated for 22 h. After centrifuging, the cell-free supernatant was reconstituted with 250 μ g Con-A in 50 μ l TC-199.

Removal of Con-A from Culture Supernatant

3 ml cell-free preincubated culture supernatant was applied on a K 16/20 column of Sephadex G-100 (Pharmacia). The bed volume of 16 ml was equilibrated in TC-199 at room temperature. Con-A was hereby removed by conjugation to the Sephadex, the low glucose concentration of TC-199 being insufficient to elute the bound mitogen.

The column was calibrated with blue dextran (MW: 2×10^6), yellow dextran (MW: 2×10^4) and vitamin B₁₂ (MW: 1357) (Gel filtration kit, Pharmacia). The column was eluted with TC-199. After passage of 4 ml eluate 9 ml samples were collected; thus the original supernatant was diluted 1/3. Molecules between approximately 10,000 and 2,000,000 daltons were recovered in the eluted samples. The mitogen-free supernatants were passed through Millipore filters (0.45 μ m pore size), tested immediately or kept frozen at -20° C.

Since even very small amounts of Con-A will inhibit leukocyte migration, three separate experiments were carried out. Human peripheral blood leukocytes were allowed to migrate under agarose after incubation in medium TC-199 $\pm$ Con-A 250 μ g/ml before and after application on Sephadex G-100 columns. As shown in Table 1, passage of Con-A-containing solutions through Sephadex G-100 columns removes all migration inhibitory activity due to Con-A.

Assay for LMIA

The indirect leukocyte migration agarose technique (ILMAT), described by Clausen (5), was employed using peripheral blood leukocytes from healthy, unrelated, human donors as migratory cells.

Venous blood in 10 ml polystyrene tubes containing 250 i.u. heparin and 2 ml 5 per cent dextran 250 in saline was allowed to sediment at 37° C for 1 h. The leukocyte-rich plasma was withdrawn, centrifuged at 220 g for 5 min and washed three times at 220 g for 5 min in Hank's balanced salt solution. 22×10^6

TABLE 1.
Leukocyte Migration Areas measured by Projection Microscope.

Supernatant	Elution on Sephadex	Migration areas ($\text{cm}^2 \pm \text{SD}$)			Exp. no. III	Mean
		I	II			
TC-199	—	50.9 ± 2.4	52.5 ± 2.3	52.1 ± 2.6		51.8
	+	50.2 ± 1.9	51.1 ± 1.9	50.1 ± 3.9		50.5
TC-199 + Con-A 250 $\mu\text{g}/\text{ml}$	—	0	0	0		0
	$\pm$	48.8 ± 3.5	52.2 ± 3.8	54.2 ± 1.6		51.7

cells were resuspended in 90 μl culture supernatant and incubated at 37°C for $1\frac{1}{2}$ h. 7 μl aliquots of cell suspensions (1.5×10^6 cells) were poured in wells punched out in agarose medium placed on plastic petri dishes (for details see (7)). After a migration period of 20 h in 2 per cent CO_2 in atmospheric air, saturated with water vapor at 37°C , the migration areas were studied under a projection microscope and measured by planimetry.

A migration index (MI) was calculated from the following formula:

$$\text{MI} = \frac{\text{Mean of leukocyte migration areas in Con-A stimulated supernatants}}{\text{Mean of leukocyte migration areas in parallel control supernatants}}$$

The cultures were set up in quadruplicate. In 13 experiments, semi-quantitative results were obtained by application of the ILMAT on stepwise diluted supernatants. Subsequently, the greatest dilution which significantly inhibited the migration of cells was determined.

In four experiments with no measurable migration inhibition under the standard test conditions described (dilution of supernatants 1/3), the test was repeated using 6- to 12-fold concentration of the original volume of supernatant. The smallest concentration which significantly inhibited the migration of cells was thereby identified. Concentration was obtained by dialysis against distilled water for 20 h (membrane pore size 24 Å), lyophilization and reconstitution in TC-199.

Statistical Methods

Statistical analysis was performed using Student's t-test for paired comparisons. *P*-values refer to significance of difference between four areas of leukocytes migrating in Con-A-stimulated lymphocyte culture supernatants, and four areas of leukocytes migrating in control supernatants.

When testing supernatants with three different indicator cell populations (Table 3), the mean values of MI were compared using F-test for analysis of variance.

TABLE 2.
Leukocyte Migration Inhibitory Activity of Supernatants from Normal Individuals.

Experiment	Sex	Age	MI $\pm$ SD (Dilution of supernatants 1/3)	P	Conc./Dil.*	MI $\pm$ SD	P
1	♂	31	0.59 $\pm$ 0.06	< 0.05	Dil. 1/3	0.59 $\pm$ 0.06	< 0.05
2	♂	37	0.33 $\pm$ 0.07	< 0.05	Dil. 1/3	0.33 $\pm$ 0.07	< 0.05
3	♂	0	0.86 $\pm$ 0.07	< 0.05	Dil. 1/3	0.86 $\pm$ 0.07	< 0.05
4	♂	38	0.84 $\pm$ 0.05	< 0.05	Dil. 1/3	0.84 $\pm$ 0.07	< 0.05
5	♀	30	0.79 $\pm$ 0.08	< 0.05	Dil. 1/24	0.88 $\pm$ 0.04	< 0.05
6	♂	29	0.84 $\pm$ 0.05	< 0.05	Dil. 1/6	0.87 $\pm$ 0.04	< 0.05
7	♂	24	0.72 $\pm$ 0.03	< 0.05	Dil. 1/3	0.72 $\pm$ 0.03	< 0.05
8	♂	48	0.94 $\pm$ 0.05	> 0.05	—	—	—
9	♂	24	0.73 $\pm$ 0.05	< 0.05	—	—	—
10	♀	29	0.73 $\pm$ 0.04	< 0.05	—	—	—
11	♂	47	0.82 $\pm$ 0.07	< 0.05	Dil. 1/3	0.82 $\pm$ 0.07	< 0.05
12	♂	49	0.90 $\pm$ 0.04	> 0.05	Conc. $\times$ 12	0.78 $\pm$ 0.06	< 0.05
13	♀	27	0.89 $\pm$ 0.02	< 0.05	Dil. 1/3	0.89 $\pm$ 0.02	< 0.05
14	♀	30	1.16 $\pm$ 0.07	ns§	Conc. $\times$ 3	0.86 $\pm$ 0.07	< 0.05
15	♂	30	1.06 $\pm$ 0.10	ns	Conc. 1/1	0.87 $\pm$ 0.04	< 0.05
16	♂	45	0.83 $\pm$ 0.03	< 0.05	—	—	—
17	♀	27	1.09 $\pm$ 0.03	ns	Conc. $\times$ 9	0.75 $\pm$ 0.08	< 0.05

* The greatest dilution or the smallest concentration of supernatants with significant migration inhibition.

§ ns = not significant.

TABLE 3.

Leukocyte Migration Inhibitory Activity tested with Three Different Indicator Cell Populations.

Super-natant No.	Indicator cells	MI (Dilution of supernatants 1/3)				MI Mean $\pm$ SD	P
1	K.B. ♂	0.82	0.85	0.84	0.88	0.85 ± 0.02	> 0.05
	Kr. ♀	0.83	0.87	0.88	0.77	0.84 ± 0.04	
	Ri ♀	0.75	0.82	0.68	0.69	0.74 ± 0.06	
2	V.A. ♂	0.73	0.73	0.77	0.81	0.76 ± 0.03	> 0.05
	A.K. ♀	0.75	0.84	0.78	0.78	0.79 ± 0.03	
	A.B. ♀	0.80	0.71	0.80	0.70	0.75 ± 0.05	
3	T.B.S. ♂	0.79	0.83	0.86	0.84	0.83 ± 0.03	> 0.05
	V.A. ♂	0.89	0.87	0.87	0.84	0.87 ± 0.02	
	Ch. ♂	0.87	0.82	0.86	0.80	0.84 ± 0.03	

RESULTS

Individual Occurrence of LMIA

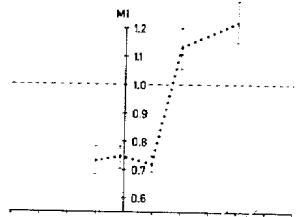
In 12 out of 17 of the normal individuals tested, significant LMIA was demonstrated under the standard test conditions described (dilution of supernatants 1/3) (Table 2). In one experiment, the activity was still present at dilution 1/24 (no 5). LMIA in four experiments could only be detected after concentration of supernatants (nos. 12, 14, 15, 17). On one occasion with no demonstrable LMIA under the standard test conditions, concentration procedures were not carried out (no 8).

In five experiments, the ILMAT was performed with 3- to 12-fold concentrated supernatants, and repeated after stepwise dilutions with culture medium (Fig. 1). Cell migration was significantly inhibited at 3- to 12-fold concentration. In two of the experiments shown in Fig. 1, a migration stimulatory activity could be demonstrated at dilutions 1/3, 1/9 and 1/12. ($P < 0.025$, t-test for paired comparisons). A similar stimulatory effect of diluted supernatants on cell migration was frequently observed in other experiments.

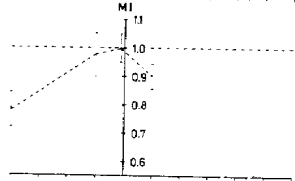
Fig. 1.

Effect on cell migration of concentration and dilution of supernatants from Con-A-stimulated human lymphocytes. Vertical lines indicates $\pm$ SD.

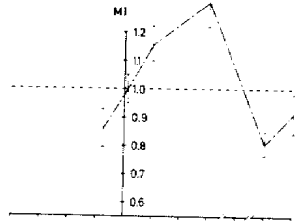
Exp. no. 7



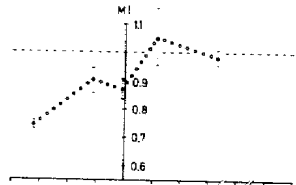
Exp no 12



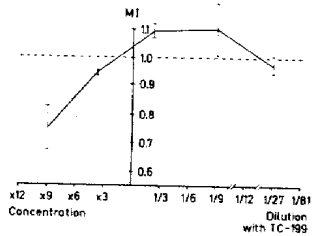
Exp no 14



Exp no 15



Exp no 17



Concentration: x12, x9, x6, x3, 1/3, 1/6, 1/9, 1/12, 1/27, 1/81
Dilution with TC-99

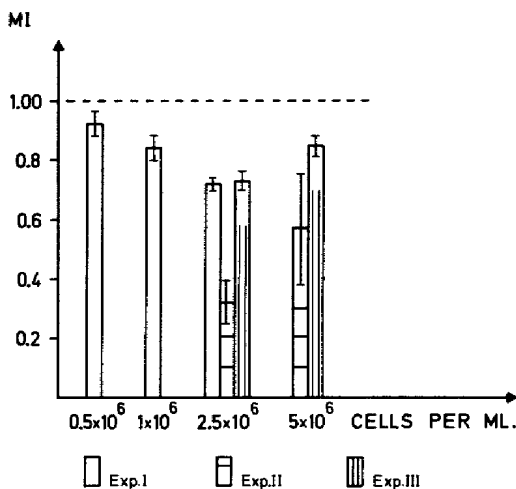


Fig. 2.

Leukocyte migration inhibitory activity tested with different concentration of mononuclear cells. Con-A conc.: $80 \mu\text{g/ml}$. Incubation time: 22 h. Vertical lines indicate $\pm$ SD.

Lymphocyte Culture Conditions

The effect on measureable LMIA release and/or production of variation in lymphocyte concentration, Con-A concentration and Con-A incubation time is shown in Figs. 2, 3 and 4, respectively.

The optimal lymphocyte concentration was 2.5×10^6 (see Fig. 2).

The concentration of Con-A stimulating optimal production of LMIA appears to be about 50 times as high as that stimulating optimal UNA synthesis (2) (Fig. 3).

In agreement with observations by several investigators, the lymphokine is detectable rapidly after activation of the lymphocytes, the activity being most pronounced after 22 h (Fig. 4).

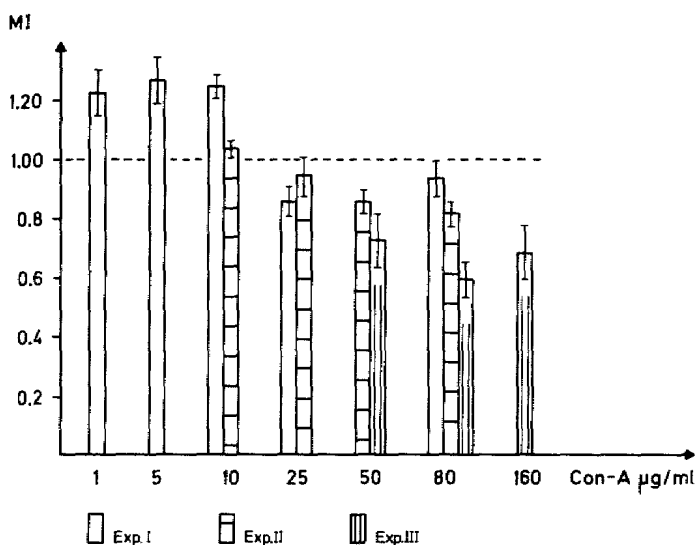


Fig. 3

Leukocyte migration inhibitory activity tested with different Con-A concentration. Mononuclear cell conc.: $2.5 \times 10^6/\text{ml}$. Incubation time: 22 h. Vertical lines indicate $\pm$ SD.

Reproducibility

The degree of reproducibility determined by standard deviations of quadruplicate tests and by simultaneous assays using different indicator cells (Table 3) appeared to be quite high. The variation between three different indicator cell populations using the same supernatant was less than 5 per cent (F-test for analysis of variance). The tests were repeated with three different supernatants with similar results (Table 3; sup. nos. 1, 2 and 3).

Consecutive determinations of LMIA in a male donor showed great stability; whereas great variation of LMIA was demonstrated in a female donor (Table 4).

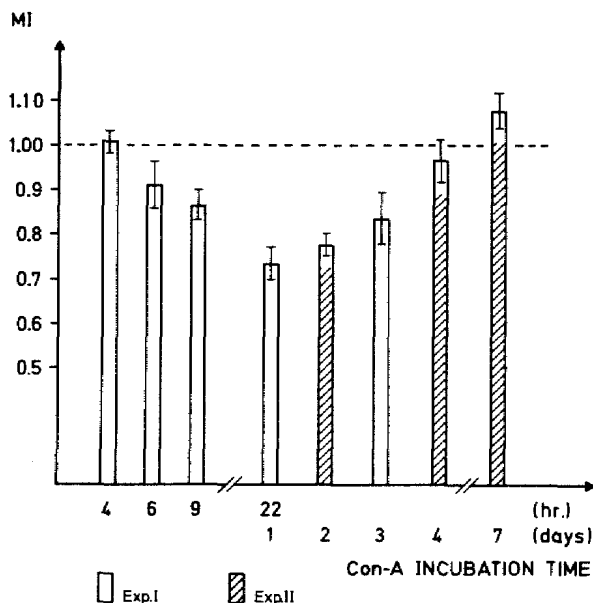


Fig. 4.

Leukocyte migration inhibitory activity tested after different Con-A incubation time. Mononuclear cell conc.: $2.5 \times 10^6/\text{ml}$. Con-A conc.: $80 \mu\text{g}/\text{ml}$. Vertical lines indicate $\pm$ SD.

Factor Stability

Fig. 5 demonstrates the disappearance of detectable LMIA in supernatants kept frozen at -20°C . The experiments were performed using three healthy donors (Case nos. 1, 2 and 3, respectively), and three patients suffering from cancer of the mammary gland with disseminated metastases (no. 4), Henoch-Schönlein purpura (no. 5) and sarcoidosis (no. 6).

Interestingly, the Con-A-induced LMIA was very pronounced and factor stability very great for the patient with sarcoidosis. The patient showed negative skin reactions to purified protein derivative of tuberculin (PPD), and when

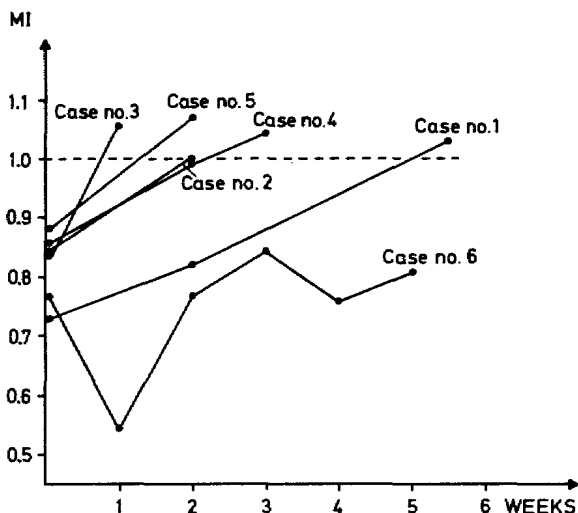


Fig. 5.

Leukocyte migration inhibitory activity stability of supernatants stored at -20°C .

the patient's lymphocytes were incubated with PPD, no LMIA could be demonstrated.

DISCUSSION

The study demonstrates that Con-A as a non-specific stimulant can induce release and/or synthesis of LMIA from peripheral human lymphocytes, and describes a standardized technique for unspecific production and semi-quantitative detection of human LMIA.

With the experimental system employed, a considerable individual variation was found in the Con-A-induced LMIA release from separated, normal peripheral blood lymphocytes. Under identical procedures, the amounts of LMIA of normal persons varied by a factor of about 300. LMIA-levels measured at different times in a male individual were more constant. A female control showed variations which were re-

TABLE 4.
Leukocyte Migration Inhibitory Activity tested at Different Times in a Healthy Male and a Healthy Female Adult.

Donor	Sex	Age	Date	Indicator cells	MI $\pm$ SD (Dilution of supernatants 1/3)	P	Conc./Dil.*	MI $\pm$ SD	P
K.B.	♂	30	8. Jan.	Kr. ♀	0.85 $\pm$ 0.06	< 0.05	Dil. 1/3	0.85 $\pm$ 0.06	< 0.05
			7. Mar.	D.L. ♂	0.84 $\pm$ 0.05	< 0.05	Dil. 1/6	0.87 $\pm$ 0.04	< 0.05
			5. Aug.	J.H. ♂	0.84 $\pm$ 0.05	< 0.05	Dil. 1/3	0.84 $\pm$ 0.05	< 0.05
L.M.	♀	30	7. Mar.	D.L. ♂	0.79 $\pm$ 0.08	< 0.05	Dil. 1/24	0.88 $\pm$ 0.04	< 0.05
			19. Mar.	Kr. ♀	0.86 $\pm$ 0.02	< 0.05	Dil. 1/6	0.90 $\pm$ 0.03	< 0.05
			4. Apr.	Du. ♀	1.08 $\pm$ 0.02	ns§	N.D.†		
			25. July	E.M. ♂	1.16 $\pm$ 0.07	ns	Conc. $\times$ 3	0.86 $\pm$ 0.07	< 0.05
			31. July	G.B. ♂	0.96 $\pm$ 0.05	ns	N.D.		

* The greatest dilution or the smallest concentration of supernatants with significant migration inhibition.

§ ns = not significant.

† N.D. = not determined.

peatedly associated in time with the menstrual cycle. Clearly, a more sufficient description of the normal occurrence and variations of Con-A-released LMIA is needed, especially since an investigation of this lymphocytic capacity is of interest in clinical conditions. The immunological state and function of the lymphocyte donor seems to be related in some way to the amount of LMIA developed after unspecific lymphocyte stimulation. Preliminary LMIA-studies in patients with various clinical conditions have so far given results of definite interest, and examinations in selected patient series are presently being undertaken.

The optimal stimulatory Con-A concentration is high as compared to the Con-A concentration used for induction of lymphocyte transformation, and the optimal culture period is shorter. In these respects, a parallel to specific, antigen-dependent lymphocyte stimulation in the similar systems is obvious: characteristically, the ILMAT uses a high antigen concentration and short incubation time as compared with the lymphocyte transformation test. It is still not clear whether LMIA is permanently on store in lymphocytes, ready for release. The appearance of antigen-induced LMIA as early as 4 h after stimulation (6) speaks somewhat in favour of pre-formed substance release; but, if so, the release seems dependent on protein synthesis (8, 11). Since a Con-A stimulation period of 24 h shows a gradual increase of LMIA, a continuous synthesis of active substance is highly possible. Yet the LMIA release does not require DNA synthesis, thus being independent of cell proliferation (4, 15).

The optimal concentration for Con-A dependent LMIA production in 24 h Con-A-containing tissue cultures was 2.5×10^6 lymphocytes per ml. Increase of the lymphocyte density in the medium resulted in a lower yield of LMIA.

The necessary removal of biologically active traces of Con-A before measuring the LMIA was efficiently accomplished by passage of the supernatants through columns of Sephadex G-100, which has a sufficiently high absorption affinity for Con-A. Since Con-A causes migration inhibition of

leukocyte cultures, this important technical point was carefully evaluated in each case by parallel control experiments with comparable, Con-A-containing, lymphocyte-free supernatants.

The *in vitro* measurement of LMIA in the second step of the technique is dependent on indicator cells prepared from unrelated, peripheral blood leukocytes. Comparison of the LMIA-sensitivity of identically prepared indicator cell populations from different healthy adults showed insignificant differences; and the indicator cells, therefore, do not appear to represent a factor of significant variability. In contrast to the similar ILMAT for detection of antigen-dependent LMIA, the previous sensitization of the indicator cell donor can be disregarded in principle.

Using the agarose technique, the standard deviations of quadruplicate cell migration areas were repeatedly very small. This is in contrast to the capillary tube technique where larger variations are encountered; with the agarose technique employed in the present study, the limit of statistically significant migration inhibition is approximately 10 per cent (Table 2).

The selection of dissolved Con-A as the lymphocyte stimulator in the present study was made from considerations of this plant mitogen in solution as a highly active stimulator of T-lymphocytes (2). None of the plant mitogens presently in use in lymphocyte research, however, seems to be an exclusive stimulator of either B- or T-lymphocytes, and even Con-A in solution may to some extent induce lymphokine release also from B-lymphocytes (2). In the 24 h Con-A stimulation period, the entire lymphocyte population of the incubate may be stimulated, either by Con-A, by lymphokines from stimulated cells, or both. This may explain why the Con-A-induced LMIA release is more comprehensive than the comparable antigen-induced LMIA release. Rocklin (16, 17) found two distinct migration inhibition factors in supernatants from antigen-stimulated, human lymphocytes:

- 1) MIF (23,000 D), detectable by guinea pig macrophage migration inhibition, was released equally well from B- and T-lymphocytes, and

- 2) LIF (69,000 D), detectable by human leukocyte migration inhibition, seemed to be prevalently a T-lymphocyte product.

If this T- and B-cell related distinction between two biochemically separable, human lymphokines is true also of the products due to antigen-independent Con-A stimulation, the use of human leukocytes as indicator cells, as in the present study, reveals prevalently LMIA released from T-lymphocytes. However, this is assumed only from analogy, and the question needs further exploration with purified T- and B-lymphocyte populations.

A technique for quantitative estimation of T-lymphocyte products synthesized and/or released immediately after Con-A stimulation will serve two main purposes: 1) It can be used for monitoring the lymphokine synthesis/release capacity of a lymphocyte population responding to a well-defined, standardized, non-specific stimulus, and 2) If identical lymphocyte populations in parallel cultures are exposed to specific antigen and to Con-A, a comparison might elucidate the role of non-specific, lymphocyte-dependent amplification in lymphokine assays.

In the guinea pig (14) and in man (3), the LMIA-yield from Con-A stimulated lymphocytes is about 10 times higher than the yield from a comparable, sensitive lymphocyte population stimulated with antigen. The method, therefore, will be useful for production of more substantial amounts of LMIA for purification and analysis. The ILMAT in its present modification seems a valuable tool for *in vitro* assessment of biological activity in fractions and products developed in the course of such efforts.

SUMMARY

Human venous blood lymphocytes, incubated for 22 h in serum-free culture medium with the plant mitogen concanavalin A (Con-A), elaborated products, which inhibited the

migration of human buffy coat cells under agarose. Con-A was removed by applying the supernatants on small Sephadex G-100 columns. The leukocyte migration inhibitory activity (LMIA) was tested in a semi-quantitative modification of the indirect leukocyte migration agarose technique, which is described. Lymphokine activity, demonstrable as early as 9 h after activation of lymphocytes, was most pronounced after 22 h.

Significant LMIA was demonstrated in 12 of 17 normal individuals at standard dilution of culture supernatants 1/3. In 9 of the 12 experiments, assays of LMIA were carried out on stepwise diluted supernatants with detection of the greatest dilution with significant LMIA. In four experiments LMIA could only be detected after 3- to 12-fold concentrations of supernatants. Considerable individual variation was found, the amounts of LMIA varying by a factor of about 300. The reproducibility appeared to be quite high, but the factor stability of supernatants stored at -20°C was surprisingly low.

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