The Production of Migration Inhibitory Factor by Nonimmune Guinea Pig Lymphoid Cells Incubated with Concanavalin A[1] (36780)

RONALD PELLEY[2] AND HOWARD J. SCHWARTZ
(Introduced by Oscard D. Ratnoff)
Department of Medicine, Case Western Reserve University School of Medicine and University Hospitals, Cleveland, Ohio 44106

Incubation of specific antigen with immune lymphocytes results in the appearance in the culture medium of several biologically active materials which have been implicated as effector materials for delayed hypersensitivity reactions (1). One of these, migration inhibitory factor (MIF), inhibits the normal migration of guinea pig peritoneal macrophages from capillary tubes (1). This phenomenon has been shown to be immunochemically specific (2, 3). MIF production does not occur when a variety of metabolic inhibitors are added to the reaction mixture (4).

It has recently been found that immunologically nonspecific production of lymphotoxin, MIF and skin reactive factor occurs when nonimmune lymphoid cells are incubated with the plant mitogen concanavalin A (Con A) (5–12). This paper presents a more detailed presentation of our previous work (7) and that of Pick *et al.* (8) regarding MIF production by nonimmunized lymphoid cells stimulated by Con A (7). The involvement of the chemical binding specificity of Con A in eliciting macrophage migration inhibition was studied by the use of methyl-α-D-mannoside—a specific inhibitor of Con A binding (16). The known inhibitory effect of puromycin on both protein synthesis and antigen induced MIF production was used to compare Con A-induced and antigen-induced migration inhibition (4). The fiinding of MIF-like activity seen by Pick *et al.* (8) in fall-through peak fractions of gel-filtered, Con A-stimulated lymphoid supernates was examined in the light of studies using radiolabeled Con A. Finally we demonstrated the quantitative absence of Con A in mitogen-induced, column-separated MIF.

Materials and Methods. Reagents. Concanavalin A (Calbiochem) was labeled with ^{125}I (International Chemical & Nuclear Corp.) by the chloramine-T method (13) and then further purified by the method of Agrawal and Goldstein (14) by absorption onto Sephadex G-75 in 1 *M* NaCl and elution with 0.10 *M* glucose in 1 *M* NaCl. Methyl-α-mannoside (Pfanstehl) was recrystallized once prior to use.

Tissue culture. Inguinal, cervical, auricular, popliteal, and axillary lymph nodes were removed from unimmunized random-bred female albino guinea pigs and teased with forceps into cold Hanks' balanced salts (Hanks' BSS) solution containing 50 mU/ml penicillin, 50 μg/ml streptomycin, and 1 U/ml heparin. The capsule of the spleen was removed and cells were teased into Hanks' BSS. The resultant cell suspensions were filtered through four thicknesses of sterile cotton gauze. The cells were then washed three times in Hanks' BSS and cultured at a concentration of 1×10^7 cell/ml in stationary Falcon 16 × 150 mm plastic culture tubes at 37°. Cultures were supplemented with 20% normal guinea pig serum (Grand Island Biological Company, NY). The culture medium used was Eagle's minimal essential medium with 2 m*M* glutamine/ml, 50 units penicillin/ml, and 50 μg/ml streptomycin (MEM). Sterile ^{125}I labeled Con A in MEM was added to cells in the stimulated group. An equal volume of MEM was added

[1] Supported by American Cancer Society Grant No. T-512, and in part by U.S. Public Health Service Grant No. AM-01005.

[2] Present Address: Department of Microbiology, Case Western Reserve University.

to cells in the control group. After 24 hr in tissue culture the tubes were centrifuged at 900 rpm for 15 min, the supernatants harvested and then centrifuged at 12,000 rpm for 20 min, supernatants were assayed for radioactivity (cpm) in a Packard Autogamma counter, and then stored at −70°. The centrifuged cell pellet was washed once in Hanks' BSS, transferred to a Packard 15 × 150 mm tube and ^{125}I radioactivity was determined. The binding was calculated:

$$\% \text{ Con A bound to cells} = \frac{^{125}\text{I cpm in pellet}}{\dfrac{\text{sp act } ^{125}\text{I-Con A (cpm}/\mu\text{g)}}{\text{Con A } (\mu\text{g}) \text{ added to tube}}} \times 100.$$

Macrophage migration. Peritoneal exudates were induced in normal, nonimmune guinea pigs by an intraperitoneal injection of 30 ml sterile light mineral oil. Three days later the animals were exsanguinated by cardiac puncture. Cold heparinized (10 units/ml) saline solution (30 ml) was injected intraperitoneally, the abdomen was kneaded gently and exudate then was harvested. The peritoneal exudate (PE) cells were separated by centrifugation at 1200 rpm, washed thrice with cold Hanks' BSS, and then reconstituted to 4 × 10^7 cells/ml in tissue culture medium 199 containing 20% normal guinea pig serum (Grand Island Biological Company, NY), 200 U/ml penicillin and 40 μg/ml streptomycin (TCM-199). Capillary tubes were filled with the cell suspension, sealed with Crito-seal, (Biological Research Inc.), and centrifuged. The tubes were cut at the cell–fluid interphase, and the portion containing the cells placed in a Mackaness-type chamber, two per chamber (2). When assaying tissue culture media or column fractions for MIF activity, 0.5 ml TCM-199 and 0.5 ml of the sample to be assayed were added to the chamber. In experiments on the direct effect of Con A upon migration, the reagents were made up to the appropriate concentration in TCM-199 and 1 ml was added to the PE cells in the migration chamber.

Chambers were incubated for 24 hr at 37° and the area of migration was measured by planimetry. The average area of migration for each preparation, based on duplicate determination, was used to calculate the percentage inhibition as follows:

$$\% \text{ inhibition} = \frac{\text{control} - \text{experimental}}{\text{control}} \times 100.$$

TABLE I. Binding of Con A to Guinea Pig Lymphocytes.[a]

Conc of con A in incubation mixture (μg/ml)	Con A bound to cells after 24 hr		No. of cell preparations
	%	(μg)	
	In serum-free media		
6	40	2.5	23
10	32	3.3	6
12	35	4.3	11
24	22	5.2	11
	In media supplemented with 20% guinea pig serum		
10	18	1.8	6

[a] ^{125}I-Concanavalin A incubated with 1 × 10^7 guinea pig lymphoid cells at 37° for 24 hr. Incubation mixture centrifuged, cells washed once, then ^{125}I cpm assayed. Binding assays were done in duplicate.

Results. Con A binding. It was found that ^{125}I-Con A binds to guinea pig lymphocytes after incubation for 24 hr in tissue culture (Table I). This binding was most pronounced in media free of sera or glycoproteins. In the absence of serum the binding was dose-related from 6 to 12 μg/ml. Addition of serum to the media reduced the degree of Con A lymphocyte binding. Thus, with 10 μg of Con A, approximately 50% less binding was observed in the serum-supplemented media.

Effect of Con A upon nonimmune peritoneal exudate cell migration. Capillary tubes containing oil induced peritoneal exudate cells were incubated in migration chambers containing TCM-199 with varying doses of concanavalin A. As shown in Table II, inhibition of migration was obtained with concanavalin A at doses of 6 μg/ml or higher. The data indicates that inhibition of migration

TABLE II. Effect of Con A Upon Migration of Nonimmune Peritoneal Exudate Cells.[a]

Dose of concanavalin (μg/ml)	No. of expts.	Inhibition of migration (%)
None		0
1	5	0
6	6	51
10	11	64
25	10	87

[a] Con A added directly to migration chambers.

increases as a function of the amount of concanavalin A. Maximal inhibition was obtained at 25 μg/ml concanavalin A, the highest dose tested.

Effect of methyl-α-D-mannoside. The simultaneous addition to the chambers of concanavalin A with 0.1 *M* methyl-α-D-mannoside interfered with the concanavalin A-induced inhibition of macrophage migration. This blocking effect was also dose related, as can be seen in Table III. At no point did 0.1 *M* methyl-α-D-mannoside completely reverse the inhibition of migration caused by concanavalin A. Higher doses of methyl-α-D-mannoside could not be utilized because of its inherent toxicity.

Effect of puromycin. In order to determine whether active protein synthesis was required for the inhibition of macrophage migration by Con A, the effect of puromycin in the system was assessed. Although puromycin itself can inhibit migration, it did reverse the effect of concanavalin A (Table IV) on macrophage migration. (The data were calculated with puromycin in the absence of concanavalin A as the control value, *i.e.*, as if the puromycin had no inhibitory effect.) The stimulated cells produced an MIF activity which was 20% above the effect of Con A added to unstimulated supernatants as controls.

TABLE III. The Effect of Methyl-α-mannoside on the In-Chamber Inhibition of Migration by Concanavalin A.

Dose of concanavalin A (μg/ml)	Methyl-α-D-mannoside (*M*)	Inhibition of migration (%)
6	0	51
6	0.1	21
10	0	64
10	0.1	28
25	0	87
25	0.1	44
0	0	0
0	0.1	10

Demonstration of MIF in supernates from Con A-stimulated lymphoid cell cultures. Since some of these effects might be due to direct effects of Con A on macrophages, the following experiments were done. Nonimmune guinea pig lymph node cells were cultured in the presence of varying doses of ^{125}I-Con A for 24 hr, and the resulting supernates were harvested and assayed for MIF activity. Since Con A by itself inhibits macrophage migration (Table II) the following control was used. Cells were cultured in medium alone, centrifuged, and the resulting supernate "reconstituted" with the amount of Con A found in the supernate of ^{125}I-Con A-incubated cells. The amount of Con A in the supernate was determined by assay of radioactivity. Con A "reconstitution" was done just prior to addition of the tissue culture fluid to the migration chamber. The data in Table V indicate that the Con A causes production of MIF by stimulated lymph node cells; the migration inhibition is not due to Con A itself.

TABLE IV. The Effect of Puromycin on the In-Chamber Inhibition of Macrophage Migration by Concanavalin A.

Dose of concanavalin A (μg/ml)	Dose of puromycin (μg/ml)	Inhibition of migration (%)
0	0	0
10	0	62
10	25	23
25	0	87
25	25	52
0	25	48

Gel filtration of MIF. In order to further demonstrate that this MIF activity was not Con A or a Con A breakdown product, the

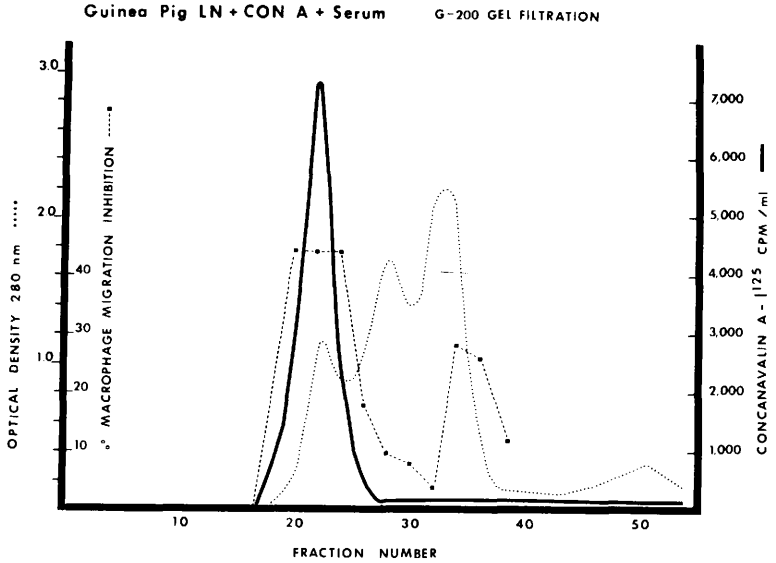

FIG. 1. MIF fractionated on G-200 Sephadex. Supernate (12 ml) from lymph node cells stimulated by Con A in serum-supplemented media was concentrated by pressure dialysis in an Amicon cell to a volume of 0.7 ml and applied to a 1.5 × 90 cm Sephadex G-200 column. The column was run at 3 ml/hr at 4° and hourly fractions were collected. (. . . .) Protein determined by OD 280 nm. (—) Con A found in column fractions from Con A-incubated cell supernates as determined by ^{125}I radioactivity. Alternate fractions were assayed after a 1:1 dilution in TCM-199. (- - -) Percentage inhibition of macrophage migration (Con A-incubated cell supernate column fractions vs fractions from columns of unstimulated cell supernates). Note two peaks of MIF activity—one corresponding with the presence of Con A (Fractions 18–26) and another (Fractions 32–38) peak free of Con A. The data is the average of two runs from two separate pools of six animals each.

following experiments were done. Tissue culture supernates were concentrated by ultrafiltration through an UM-10 Amicon membrane (American Corp.) and fractionated by Sephadex G-200 gel filtration. Sephadex G-100 and G-200 tightly absorb free Con A (14). Lymph node cells (1 × 10^7 cells/ml) were stimulated with 6 μg ^{125}I-Con A/ml in the presence of 20% normal guinea pig serum for 24 hr, the supernatants harvested, concentrated and gel filtered. Two zones of MIF activity were found, one (Tubes 34–36) eluting after albumin (Tubes 32–35), and free of ^{125}I activity. MIF activity was also found in the fall-through region. In this region was also found all of the ^{125}I-Con A that was eluted from the column (Fig. 1). Con A can form complexes with many serum glycoproteins (10, 15). This macromolecular Con A comprised 80% of the Con A applied to the column. (87,000/109,000 cpm) When Con A is incubated with lymphocytes in media unsupplemented with high molecular weight substances and the supernate filtered through G-200 less than 3% of the Con A elutes in the fall-through region.

TABLE V. Migration Inhibition of Nonimmune Guinea Pig Peritoneal Exudate Cells by Lymph Node Tissue Culture (TC) Supernates.[a]

Concn of Con A (μg/ml)	Migration inhibition (%)
3	20
6	26
12	44
24	25

[a] % MIF = 100 — [(migration in presence of Con A stimulated TC supernates)/(migration in presence of Con A + unstimulated TC supernates)].

The ^{125}I-Con A used in these experiments

TABLE VI. Production of MIF by Nonimmune Guinea Pig Spleen Cells Stimulated by Con A and Fractionated on G-200 Sephadex.

	Fall-through region[a]	7s region[a]	Post-albumin region[a]
	Spleen cells cultured in MEM + serum		
Presence of Con A	+	—	—
Inhibition of migration[b] (%)	40	0	20
	Spleen cells cultured in MEM alone		
Con A	—	—	—
Inhibition of migration[b] (%)	0	7	26

[a] Similar tube positions to Fig. 1.

[b] As calculated against equivalent column fractions from unstimulated cultures.

had a specific activity of 1000 cpm/μg. It is therefore unlikely that enough Con A was present in the 4–5 S region to cause the MIF effect seen: *i.e.*, $<$ 50 cpm radioactivity $= <$ 50 ng Con A. Amounts of less than 1 μg of Con A are without direct effect upon migration of nonimmune peritoneal exudate cells.

Production of MIF by Con A-stimulated spleen cells. Guinea pig spleen cells cultured in media with concanavalin A and serum supplement show significant production of MIF (Table VI). Spleen cells incubated with concanavalin A without serum supplementation also produced significant amounts of MIF.

Discussion. The jackbean globulin concanavalin A has been prepared as a homogenous protein with known chemical specificities (14). It reacts specifically with sugars containing the D-arabinopyranoside structure at C3, C4, and C6, and can readily be dissociated from complexes with glycoproteins or polysaccharides by such simple chemicals as methyl-α-D-mannoside (16). Concanavalin A also reacts with lymphocytes to stimulate the incorporation of tritiated thymidine (16) and the secretion of skin reactive factor (10–12) and lymphotoxin (6, 7).

In this paper we have demonstrated that the addition of Con A to migration chambers inhibits the migration of normal peritoneal exudate cells, and stimulated the production of MIF by nonimmune lymphocytes. The α-1,4-arabinopyranoside structure would seem to be as critical for MIF production as it is for lymphocyte stimulation, since 0.1 *M* methyl-α-D-mannoside significantly inhibited this effect. Development of MIF activity was abolished by concomitant addition of puromycin and Con A to the chambers. Using similar methods, antigen induced MIF production can also be inhibited by puromycin (4). That an MIF distinct from Con A was produced was demonstrated by the fact that Con A stimulation of lymph node cells in serum-supplemented media resulted in the production of an MIF eluting after albumin on Sephadex G-200 gel filtration; in this property this MIF resembles MIF induced by incubation of antigen with antigen-reactive cells (17).

Pick *et al.* (8) have also produced MIF by Con A stimulation of peritoneal exudate lymphocytes or lymph node cells. In their experiments, MIF-like material was detected in the fall-through peak of gel-filtered supernates from stimulated lymphocytes. This peak may have contained significant Con A, as we have demonstrated by our ^{125}I labeling experiments. MIF activity was also found in pooled fractions eluting with hemoglobin. Our present studies show that Con A induced MIF elutes with and after albumin on gel filtration and is free of ^{125}I-Con A (50 ng/ml). It is interesting to note that little soluble complex Con A–glycoprotein is formed on incubation of spleen cells with Con A in media without serum supplement and little MIF-like activity is found in fall-through peak material.

It is now apparent that production of a variety of mediators can be mitogen induced. Con A-stimulated MIF has a molecular weight close to that of antigen-stimulated MIF (less than that of albumin) (17). PHA-induced mouse lymphotoxin shares many

properties with antigen (mixed lymphocyte culture) induced mouse lymphotoxin (LT) (19, 20). Con A and pokeweed mitogen also can cause production of LT by nonimmune cells (5, 6, 22). Skin reactive factor (SRF) can be induced by Con A stimulation of lymphocytes (8, 10, 11). It has a molecular weight similar to that of the antigen-stimulated material (23). Con A-stimulated SRF is capable of faithfully reproducing the tuberculin skin lesion (12). Pokeweed mitogen is capable of inducing a tuberculin like lesion *in vivo* upon injection of the mitogen into the skin of nonimmune animals (21). Thus, antigen and mitogen-induced mediators may be identical (9, 24).

Summary. Migration inhibitory factor (MIF) is produced upon concanavalin A (Con A) stimulation of nonimmune lymphoid cells. This production is specific for the α-1, 4-arabinopyranoside affinity of the Con A as demonstrated by the inhibitory effect of methyl-α-D-mannoside. Con A-induced MIF synthesis is puromycin sensitive. Con A-induced MIF can be separated from Con A by column chromatography through Sephadex G-200. This mitogen-induced mediator seems to have approximately the same molecular weight as the antigen-induced MIF as measured by gel filtration. MIF can be produced by lymph node cells in the presence of serum supplemented media. Spleen cells do not require serum-supplemented media to produce Con A-induced MIF.

1. Lawrence, H. S., and Landy, M., eds., "Mediators of Cellular Immunity." Academic Press, New York (1969).

2. David, J. R., Al-Askari, S., Lawrence, H. S., and Thomas, L., J. Immunol. **93**, 264 (1964).

3. David, J. R., and Schlossman, S. F., J. Exp. Med. **128**, 1451 (1968).

4. David, J. R., J. Exp. Med. **122**, 1125 (1965).

5. Stavy, L., Treves, A. J., and Feldman, M., Nature (London) **232**, 56 (1971).

6. Schwartz, H. J., and Wilson, F., Amer. J. Pathol. **64**, 295 (1971).

7. Schwartz, H. J., Pelley, R. P., and Leon, M. A., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 360 (1970).

8. Pick, E., Brostoff, J., Krejci, J., and Turk, J. L., Cell. Immunol. **1**, 92 (1970).

9. Coyne, J. A., Remold, H. G., and David, J. R., Fed. Proc., Fed. Amer. Soc. Exp. Med. **30**, 647 Abstr. (1971).

10. Schwartz, H. J., Leon, M. A., and Pelley, R. P., J. Immunol. **104**, 265 (1970).

11. Pick, E., Krejci, J., and Turk, J. L., Nature (London) **225**, 236 (1970).

12. Schwartz, H. J., Catanzaro, P. J., and Leon, M. A., Amer. J. Pathol. **63**, 443 (1971).

13. McConahey, P., and Dixon, F., Int. Arch. Allergy Appl. Immunol. **29**, 185 (1966).

14. Agrawal, B. B. L., and Goldstein, I. J., Biochem. J. **96**, 23c (1965).

15. Leon, M. A., Science **158**, 1325 (1967).

16. Powell, A. E., and Leon, M. A., Exp. Cell. Res. **62**, 315 (1970).

17. Remold, H. G., Katz, A. B., Haber, E., and David, J. R., Cell. Immunol. **1**, 133 (1970).

18. Pick, E., Krejci, J., Cech, K., and Turk, J. L., Immunology **17**, 741 (1969).

19. Kolb, W. P., and Granger, G. A., Cell. Immunol. **1**, 122 (1970).

20. Granger, G., *in* "*In vitro* Methods in Cell-Mediated Immunity" (B. R. Bloom, and P. R. Glade, eds.), p. 572. Academic Press, New York (1971).

21. Astaldi, G., Burgio, G. R., Gemova, R., Curtoni, E., and Girando, L. R., RES, J. Reticuloendothel. Soc. **7**, 126 (1970).

22. Williams, T. W., and Granger, G. A., J. Immunol. **103**, 170 (1969).

23. Bennet, B., and Bloom, B., Proc. Nat. Acad. Sci. U.S.A. **59**, 756 (1968).

24. David, J. R., *in* "Progress in Immunology" (B. Amos, ed.), p. 399. Academic Press, New York (1972).

Received Apr. 18, 1972. P.S.E.B.M., 1972, Vol. 141.