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INHIBITION OF LEUKOCYTE MIGRATION BY THE AGAROSE PLATE TECHNIQUE

*Application to Antigen from Candida albicans and
Fusobacterium nucleatum*

By

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Specific cell-mediated immunity to microbial antigens can be detected *in vitro* by several methods including the leukocyte migration capillary tube test (LMCT) described by Söborg & Bendixen (18) and its modification, the leukocyte migration agarose test (LMAT) described by Clausen (8). The inhibition of leukocyte migration was produced when antigen interacted with sensitized peripheral blood leukocytes and correlated to delayed hypersensitive skin test reactivity (5, 8, 22). Recently, a leukocyte migration inhibitory factor (LIF) was isolated after antigen stimulation of sensitive human B and T lymphocytes (7, 16); this substance, which was distinct from MIF, inhibited the migration of human polymorphonuclear leukocytes, but not monocytes. In the LMAT the primary migrating cells are polymorphonuclear leukocytes and the migration of these cells was inhibited by LIF (12).

We have shown that the antigens from the typical dental plaque bacteria *Actinomyces viscosus*, *Fusobacterium nucleatum*, *Bacteroides melaninogenicus*, and *Veillonella parvula* could influence the migration of peripheral blood leukocytes in the agarose test system (5, 6). However, the results were not uniform. Thus, *V. parvula* antigen induced considerably stronger migration inhibition in patients with periodontal dis-

ease as compared with controls than did any of the other antigens. The results suggested that the degree of migration inhibition was dependent on the concentration of protein in the antigen preparation. We have also failed to demonstrate migration inhibition to soluble *Candida* antigen in subjects with a positive delayed hypersensitive skin reaction and a significant migration inhibition response to particulate antigen (4).

In this report we extend our initial observations to show that leukocyte migration inhibition, dependent on an intact protein synthesis in the migrating cell population, is achieved when concentrated solutions of *F. nucleatum* or *C. albicans* antigen are employed.

MATERIAL AND METHODS

The material comprised healthy adults under no medical treatment which is supposed to influence cellular immunity. All persons had natural teeth and slight periodontal inflammation, without pathologic pocket formation.

Agarose plate migration technique. The direct LMAT according to Clausen (8, 9) was carried out after the modification of Mygind & Stenbjerg (15) using peripheral blood leukocytes as migratory cells. Venous blood was collected in 10-ml polystyrol tubes, each containing 250 iu of preservative-free heparin (Novo, Bagsvaerd, Denmark) and 2 ml 5 per cent dextran 250 (Dextran T 250, Pharmacia, Uppsala, Sweden) in 0.9 per cent NaCl. After sedimentation for $\frac{1}{2}$ h at 37° C, the leukocyte-rich plasma was withdrawn, centrifuged at 160 g for 5 min, and the cells were washed 3 times in Hanks's Balanced Salt Solution by centrifugation at 160 g for 5 min. The cells were resuspended in Hepes-buffered TC 199 (Difco Laboratories, Detroit, Mich., U.S.A.) with 10 per cent horse serum at final concentration of 2.1×10^6 cells/ml, and small aliquots were incubated for 30 min with different antigen solutions or as controls—with saline used as diluent. Seven μ l aliquots of cell-suspension (1.5×10^6 cells) were introduced in wells punched out in agarose medium placed on plastic Petri dishes, and were incubated at 37° C for 21 h. The migration areas were studied in a Dokumator DC II (VEB Carl Zeiss, Jena, DDR) and measured (11). The cultures were set up in quadruplicate and the mean area of identically treated migration cultures—minus the area of the well—was calculated. A migration index was calculated from the formula:

$$MI = \frac{\text{Migration area with antigen}}{\text{Migration area without antigen}}$$

Puromycin. Antigen-containing and control cultures were parallelly incubated with or without puromycin. Puromycin (Calbiochem., U.S.A.) was dissolved in TC 199, with 10 per cent horse serum and antibiotics, at a concentration of 1 mg/ml, and was stored at -20°C until used. The final concentration of puromycin in the cultures was $12.5\text{ }\mu\text{g/ml}$ (15). The cell suspensions were incubated with puromycin at 37°C for 15 min prior to the addition of antigen. A puromycin migration index was calculated from the formula:

$$\text{MI} = \frac{\text{Migration area with antigen and puromycin}}{\text{Migration area with puromycin}}$$

Preparation of antigens. An antigen extract was prepared from *F. nucleatum*, as described previously (6), and was kindly donated by Dr. J. Kelstrup, Dept. of Microbiology, Royal Dental College, Århus, Denmark. *F. nucleatum* is known to be a predominant microorganism of the gingival crevice area and is involved in the pathogenesis of periodontal disease (17). Briefly, a concentrated suspension of bacteria was subjected to prolonged ultrasonication, and cells and debris were removed by centrifugation. Lipids were removed by ether and the preparation was made up with saline to equal a concentration of approximately 2×10^{10} bacteria/ml.

The *Candida* antigen was prepared from *C. albicans* type A (Hasenclever) according to Axelsen (1), and was kindly donated by Dr. E. Svejgaard, Dermatological Dept., Rigshospitalet University Hospital, Copenhagen, Denmark.

The antigen solutions were assayed for protein content with the Lowry procedure (13). The values found were:

<i>F. nucleatum</i> :	157.5 $\mu\text{g/ml}$
<i>C. albicans</i> :	10.0 mg/ml

Five-fold concentrated solutions of *F. nucleatum* antigen and 10-fold concentrated solutions of *C. albicans* were prepared in a Clinical Sample Concentrator, Minicon B, mol.wt. 15,000 (Amicon B.V., Oosterhout (NB), Holland). Cultures were set up parallelly with the concentrated and the original antigen preparations.

Skin testing. Cellular immunity was assayed *in vivo* by intracutaneous injection of 0.1 ml of the extract of *C. albicans* in a 1/1000 dilution. A skin induration exceeding 5 mm was considered a positive reaction.

Statistical Methods

Sample means were compared by Student's *t*-test. In parallel experiments, with or without puromycin and with different concentrations of antigen, statistical analysis was performed using Student's *t*-test for paired comparisons. Variances were analysed by means of the F-test.

CANDIDA - ANTIGEN

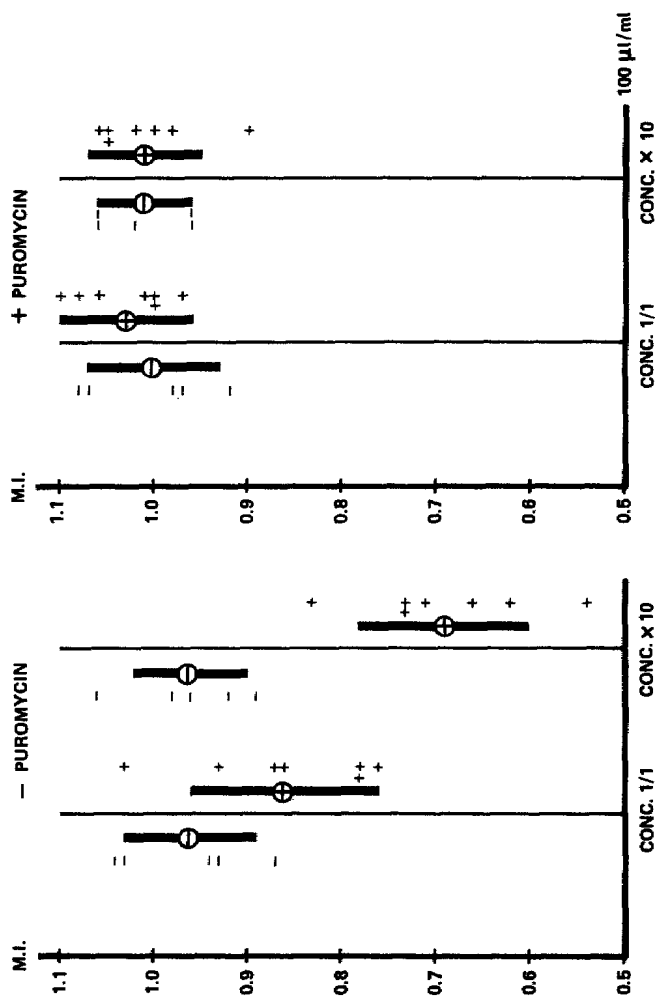


Fig. 1.

RESULTS

Fig. 1 shows the distribution of migration indices in five subjects with a negative intracutaneous reaction to candida antigen and in seven subjects with a positive reaction. With the unconcentrated candida antigen the mean value of MI was 0.96 ± 0.07 for the skin-test negative subjects and 0.86 ± 0.10 for the skin-test positive subjects; the mean values were not significantly different. With the concentrated antigen the mean values obtained were 0.96 ± 0.06 and 0.69 ± 0.09 , respectively, and were significantly different ($t = 5.1$; $P < 0.001$). In the group of skin-test negative subjects there was no difference when comparing the mean MI obtained with the unconcentrated antigen with that obtained with the concentrated antigen. In the group of skin-test positive subjects the concentrated candida antigen caused an increased inhibition of migration as compared with the unconcentrated suspension. Puromycin abolished migration inhibition by both solutions of candida antigen.

The migration response to candida antigen was studied in 22 subjects, tested with both the concentrated and the original solution of candida antigen, with or without puromycin. The mean value of MI with the unconcentrated antigen solution with or without puromycin was 1.01 ± 0.07 and 0.91 ± 0.13 , respectively; this difference was statistically significant ($t = 2.87$, $0.001 < P < 0.01$, t -test for paired comparisons).

With the concentrated antigen solution, migration inhibition was further increased in the cultures without puromycin, mean value of MI 0.77 ± 0.13 , but migration inhibition was not augmented in the cultures with puromycin, mean value of MI

Fig. 1.

Migration index (MI) in candida-positive (+) and candida-negative (—) subjects in the presence of unconcentrated and concentrated doses of candida antigen in the cultures, and with or without puromycin. The circles indicate the mean value of MI for each dose of antigen; the vertical lines represent the standard deviation.

1.02 ± 0.06 ; the difference between the migrations with and without puromycin was highly significant ($t = 6.55$, $P < 0.001$, t -test for paired comparisons).

Studies with Fusobacterial Antigen

The migration indices obtained in 10 subjects with slight periodontal inflammation are recorded in Table 1. The mean MI with or without puromycin and with either the original or the concentrated antigen solution did not differ significantly. However, the variances of the observations were different when comparing the MI of puromycin-containing cultures with those without puromycin. With the original antigen solution $F = 6.71$, $P < 0.01$, and with the concentrated solution $F = 45.03$, $P < 0.01$.

TABLE 1

Migration Indices in 10 Persons with Slight Periodontal Inflammation Tested with Antigen from Fusobacterium nucleatum.

	Doses of antigen			
	100 μ l/ml, conc. 1/1		100 μ l/ml, conc. 5 $\times$	
	— Puromycin	+ Puromycin	— Puromycin	+ Puromycin
1.	0.88	1.02	0.74	1.03
2.	1.11	0.98	1.02	1.03
3.	1.50	1.00	0.84	1.08
4.	1.30	0.86	1.36	0.93
5.	1.08	1.05	0.90	1.07
6.	0.70	0.97	0.69	0.96
7.	0.92	0.94	0.71	0.97
8.	1.16	0.99	1.38	0.92
9.	1.18	0.95	1.85	0.99
10.	1.05	1.08	0.75	0.95
Mean value	1.09	0.98	1.04	0.99
S.D.	± 0.22	± 0.08	± 0.38	± 0.06

Typical changes in MI in individual subjects caused by puromycin or a 5-fold increase in the concentration of antigen

FUSOBACTERIAL ANTIGEN

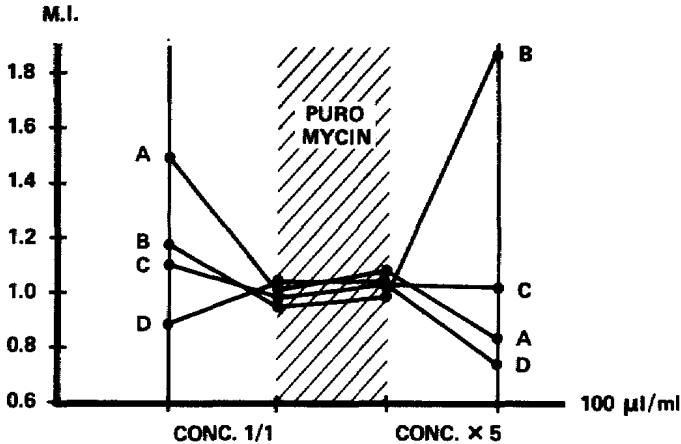


Fig. 2.

Recorded migration index (MI) from four subjects with slight periodontal inflammation tested with the direct LMAT in the presence of unconcentrated and concentrated doses of fusobacterial antigen in the cultures, and with or without puromycin.

is shown in Fig. 2. Thus, subject A showed a change from pronounced stimulation of migration to inhibition of migration; subject B showed a change from slight stimulation of migration to pronounced stimulation; in subject C, MI remained about zero; and subject D showed a change from slight inhibition of migration to more pronounced inhibition. Puromycin abolished both stimulation and inhibition of migration.

DISCUSSION

In a previous study using the LMCT it was found that migration inhibition by particulate candida antigen correlated well with the delayed hypersensitive intracutaneous reaction to candida, whereas a soluble candida antigen did not induce sig-

nificant migration inhibition in sensitive subjects (4). In the present study significant inhibition of migration was demonstrated with the LMAT in subjects with a positive intracutaneous reaction to candida antigen when the leukocytes were cultured with a 10-fold concentrated antigen solution. Furthermore, migration inhibition by both the original and the concentrated antigen was consistently abolished when puromycin was added to the cultures.

Puromycin is an inhibitor of proteinsynthesis and has been shown to prevent immunologic migration inhibition of peripheral blood leukocytes from Mantoux-positive persons, both in the macrophage migration test system, the LMCT and the LMAT (5, 10, 14). Puromycin causes some non-specific inhibition of migration in control cultures but has been shown to reveal false, probably toxic migration inhibition by high concentrations of PPD in the LMAT (15). In contrast, Bergstrand et al. (3) found that the non-specific inhibition of migration by puromycin was so pronounced in control and particularly in antigen-containing cultures that migration inhibition of sensitive leukocytes cultured with PPD and puromycin was still significant. According to the present study the mean area of the cultures with puromycin was about 10 per cent smaller than the corresponding mean area of cultures without puromycin; however, specific immunologic inhibition of migration in persons with a positive delayed hypersensitive intracutaneous reaction to candida was prevented by puromycin. In order to obtain a consistent prevention of immunologic migration inhibition it is important to preincubate the leukocytes with puromycin, prior to addition of antigen (unpublished observations).

In patients with pronounced periodontal inflammation leukocyte migration inhibition to the pathogenic microorganism, *F. nucleatum*, was found to be significant as compared with a group of edentulous controls (6). In the present study the mean values of MI for a group with slight periodontal inflammation were close to 1.00, both with the concentrated and the unconcentrated antigen solution. However, the variances

of MI were significantly larger in the cultures without puromycin, indicating that antigens from *F. nucleatum* did influence the migration of leukocytes. With the original antigen the limit of the normal range in the puromycin-containing cultures was 1.14–0.82 (95 per cent confidence limits); 50 per cent of the corresponding cultures without puromycin showed either migration inhibition or stimulation of migration. With the concentrated antigen solution the normal range of the puromycin-containing cultures was 1.11–0.87; in comparison, 80 per cent of the corresponding cultures without puromycin showed either stimulation or inhibition of migration. A distinct pattern was seen. Thus, in cultures showing stimulation of migration a 5-fold increase in the concentration of antigen resulted either in a further stimulation of migration or migration inhibition; in cultures showing slight inhibition of migration, the inhibition was augmented with the concentrated antigen solution, but stimulation of migration did not occur. These findings corroborate with studies employing the capillary tube technique with high and low concentrations of brucella antigen (19). Leukocyte migration stimulatory activity has also been demonstrated in diluted supernatants of Con-A-stimulated leukocytes whereas inhibitory activity was found in concentrated supernatants (2).

Two distinct factors which influence leukocyte migration in the agarose test system have been demonstrated, one enhancing (LEF) and one inhibiting (LIF) leukocyte migration (21); it was suggested that the proportions of the two factors might determine the net result in the leukocyte migration test. In this study stimulation of migration by antigen from *F. nucleatum* was consistently prevented by puromycin, a further evidence that stimulation of migration is an immunological reaction. It has been proposed that stimulation of migration is the effect of weaker antigenic stimulation or less sensitive lymphocytes; a temporal difference in the production of LIF and LEF; or the nature of the antigen (19, 20, 21). In the present study the patients showed only slight periodontal inflammation and relatively moderate accumulations of dental

plaque; it is likely therefore that the lymphocytes were less sensitive, which could explain that enhancement of migration rather than inhibition of migration was obtained by antigen from *F. nucleatum*. In a study on leukocyte migration inhibition by dental plaque antigen in patients with more severe periodontal inflammation, migration inhibition to antigen from *F. nucleatum* was significant, but stimulation of migration did not occur (6).

In conclusion, the present study confirms that it is important to study dose-related effects when applying the LMAT to test cell-mediated immunity to microbial antigens. The study is also in support of stimulation of migration being an immunologic reaction.

SUMMARY

Leukocyte migration inhibition by antigen from *Candida albicans* and *Fusobacterium nucleatum* was studied in man by means of the direct leukocyte migration agarose technique (LMAT). Antigens were prepared by prolonged ultrasonication and were added to the leukocyte cultures in the original as well as in the concentrated solutions.. Significant inhibition of migration with a 10-fold concentrated solution of candida antigen was demonstrated in subjects showing a positive intracutaneous reaction to candida. With the fusobacterial antigen, inhibition of migration or stimulation of migration were demonstrated in subjects with slight periodontal inflammation. A 5-fold increase in the concentration of fusobacterial antigen resulted in increased stimulation of migration or inhibition of migration. Preincubation of the leukocytes with puromycin abolished both inhibition and stimulation of migration by candidal or fusobacterial antigen. The study confirms that it is important to study dose-related effects when applying the LMAT to test cell-mediated immunity. The study is in support of stimulation of migration being an immunologic reaction.

REFERENCES

1. Axelsen, N. H. (1973): Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization to immunochemistry of *Candida albicans*. *Infect. Immun.* 7, 949-960.
2. Bendtzen, K., Andersen, V. & Bendixen, G. (1975): An *in vitro* assay of leukocyte migration inhibitory activity from human lymphocytes stimulated with concanavalin A. *Acta allergol.* 30, 133-149.
3. Bergstrand, H., Källén, B. & Nilsson, O. (1974): On the reactivity of human leukocytes to PPD in Clausen's agarose migration technique. *Acta allergol.* 29, 117-135.
4. Budtz-Jørgensen, E. (1972): Delayed hypersensitivity to *Candida albicans* in man demonstrated *in vitro*: the capillary tube migration test. *Acta allergol.* 27, 41-49.
5. Budtz-Jørgensen, E., Kelstrup, J. & Budtz-Jørgensen, A. (1976): Leukocyte migration inhibition by dental plaque antigens as indicated by the capillary tube and the agarose plate technique. *J. periodont. Res.* 11, 186-195.
6. Budtz-Jørgensen, E., Kelstrup, J., Funder-Nielsen, T. D. & Knudsen, A. M. (1976): Leukocyte migration inhibition by bacterial antigens in patients with periodontal disease. *J. periodont. Res.*, in press.
7. Chess, L., Rocklin, R. E., MacDermott, R. P., David, J. R. & Schlossman, S. F. (1975): Leukocyte inhibitory factor: Production by purified human T and B lymphocytes. *J. Immunol.* 115, 315-317.
8. Clausen, J. E. (1971): Tuberculin-induced migration inhibition of human leucocytes in agarose medium. *Acta allergol.* 26, 56-80.
9. Clausen, J. E. (1973): Comparison between capillary tube and agarose migration technique in the study of human peripheral blood leukocytes. *Acta allergol.* 28, 145-158.
10. David, J. R. (1965): Suppression of delayed hypersensitivity *in vitro* by inhibition of protein synthesis. *J. exp. Med.* 122, 1125-1134.
11. Ferraresi, R. W., Dedrick, C. T., Raffel, S. & Goihman-Yahr, M. (1969): Studies of macrophage inhibition test. I. Comparison of the skin and cell migration reactions during the course of development of delayed hypersensitivity. *J. Immunol.* 102, 852-858.
12. Hoffman, P. M., Spittler, L. E., Hsu, M. & Fudenberg, H. H. (1975): Leukocyte migration inhibition in agarose. *Cellular Immunol.* 18, 21-30.
13. Lowry, O. H., Roseborough, N. T., Farr, A. L. & Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
14. Mitchell, C. G., Smith, M. G. M., Golding, P. L., Eddleston, A. L. W. F. & Williams, R. (1972): Evaluation of the leucocyte migration test as a measure of delayed hypersensitivity in man. *Clin. exp. Immunol.* 11, 535-541.
15. Mygind, K. & Stenbjerg, S. (1974): Leucocyte migration test in agarose. The use of puromycin in disclosure of non-immunological inhibition. *Acta path. microbiol. scand. Sect. B*, 82, 277-282.

16. Rocklin, R. E. (1974): Products of activated lymphocytes: Leucocyte inhibitory factor (LIF) distinct from migration inhibition factor (MIF). *J. Immunol.* *112*, 1461-1466.
17. Socransky, S. S. (1970): Relationship of bacteria to the etiology of periodontal disease. *J. dent. Res.* *49*, 203-222.
18. Søbørg, M. & Bendixen, G. (1967): Human lymphocyte migration as a parameter of hypersensitivity. *Acta med. scand.* *181*, 247-256.
19. Søbørg, M. (1968): *In vitro* migration of peripheral, human leucocytes in cellular hypersensitivity. *Acta med. scand.* *184*, 135-145.
20. Weisbart, R. H., Cunningham, J. E., Bluestone, R. & Goldberg, L. S. (1973): A modified agarose method for detection of migration inhibitory factor and delineation of its antigen dependency. *Int. Arch. Allergy* *45*, 612-619.
21. Weisbart, R. H., Bluestone, R., Goldberg, L. S. & Pearson, C. M. (1974): Migration enhancement factor: A new lymphokine. *Proc. nat. Acad. Sci. (Wash.)* *71*, 875-879.
22. Weisbart, R. H., Isaacson, J., Bluestone, R. & Goldberg, L. S. (1975): Human polymorphonuclear leucocyte migration inhibitory factor. *Immunology* *29*, 223-230.

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