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A Simple *In Vitro* Method for Studies on Chemotaxis (38367)

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(Introduced by J. J. Munoz)
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In 1962, Boyden (1) introduced an *in vitro* method for the study of chemotaxis. In principle, the method is simple and, indeed, it is the predominant one in use today. In practice, however, the technique has some inherent shortcomings (2, 3), and is expensive as well as technically tedious.

The Boyden method has been used clinically to determine if polymorphonuclear leukocytes (PMN) from patients will respond normally to a known chemotactic stimulus. Presumably due to the above drawbacks, however, this method has had limited usefulness as a clinical tool. This is unfortunate, since abnormal PMN responses have been found to occur among patients suffering from recurrent bacterial infections (4, 5) and in some persons with pulmonary infections (6). Because of the limited testing, it is not known whether other diseases may be related to impaired chemotactic responses of PMN.

The phenomenon of leukocyte migration on the surface of a plastic Petri dish was used to develop a method for *in vivo* studies of chemotaxis. The technique allows for direct observation of migrating cells; it is technically simpler and possibly more sensitive than the Boyden chamber technique.

Materials and Methods. PMN were harvested in medium 199 (TC-199) with 0.05 *M* ethylenediamine tetraacetic acid (EDTA) pH 7.2 from the peritoneal cavity of guinea pigs previously inoculated (12–13 hr) intraperitoneally with 0.5% glycogen in saline. The cells were washed once in 50 vol of TC-199 without EDTA and finally suspended at 1–2 $\times$ 10^8 cells/ml of TC-199 plus 10% fetal calf serum (FCS).

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Two milliliters of 0.75% agarose in TC-M199 (pH 7.2) + FCS were added to each 35 $\times$ 10 mm plastic Petri dish (Falcon Plastics, Oxnard, CA). Wells were prepared with cylindrical cutters of appropriate diameters and arranged in various patterns. Most commonly, three wells, 2.4 mm in diam, were prepared with a cutoff 12 gauge hypodermic needle and arranged in a straight line, 2.4 mm apart. Agarose was removed from the wells with a capillary pipette through which slight suction was applied. In this process, it was essential to avoid introduction of air bubbles between the agarose and the Petri dish. The plates were allowed to equilibrate for 2–3 hr at 37° in an atmosphere of 5% CO_2 and 95% air saturated with water vapor. Five microliters of TC-199 + FCS were carefully added to the first well (neutral well) with a Pasteur pipette and rubber bulb. The same volume of cell suspension was similarly added to the middle well, and the test or control material to the third well. The plates were incubated under the above equilibration conditions. At various times, the migration of cells toward the test material was compared to the migration toward the negative control. The distances of migrations were measured with an ocular micrometer (microscope with 4$\times$ objective and 10$\times$ ocular lenses). When desired, the cells were permanently fixed and stained *in situ* by treating with absolute methanol overnight followed by removal of agarose and staining with Giemsa for 30 min.

To compare the agarose system with the Boyden chamber technique, a commercial chamber (Neuroprobe, Inc., Bethesda, MD) was used along with a 5 μm pore filter (Millipore Corp., Bedford, MA). The attractant side of the filter contained either TC-199 + FCS as the

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negative control or chemotactic substance diluted in TC-199 + FCS. The cell side of the filter contained a suspension of PMN, obtained as described earlier, at a concentration of 1–2 × 10^6 cells/ml. After 3 hr of incubation at 37° and 5% CO_2, the membrane was removed, fixed in methanol and stained with hematoxylin (1). The number of cells that migrated completely through the filter was counted in five high power fields and averaged.

A strain of *Escherichia coli*, as well as some known chemotactically active material produced from this organism, were generously supplied by Dr. T. Mitchell (University of Minnesota). A strain of *Staphylococcus epidermidis* was isolated as a contaminant from a skin lesion of a patient. Chemotactically active materials were produced from both of these organisms as previously described (7).

Results. Wright-stained smears of harvested white cells showed that 90–95% of the harvested cells were PMN, and vital staining (trypan blue) indicated 95–98% viability. Under the prescribed conditions for the agarose method, the cells migrated in a single plane at the interface between the agarose and Petri dish. The predominant migrating cell type was the PMN, although mononuclear leukocytes were also noted.

Table I shows the distances of migration, after 3 hr incubation of cells, toward chemotactically active materials as compared to the negative control (TC-199 + FCS). The data show good correlation with the Boyden method.

Migration rates were found to vary with time of incubation (Fig. 1). By 2 hr, the migration rate of leukocytes toward a chemotactic substance (from *E. coli*) was about 16 times that of cells randomly migrating (negative control). The migration rate of stimulated cells rapidly declined after 2 hr and by 22 hr actually fell below that of the negative control.

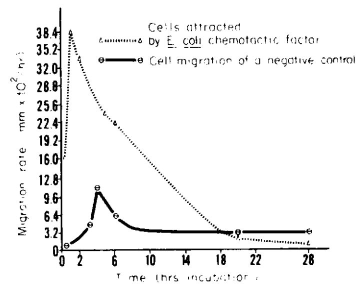

FIG. 1. Migration rates of leukocytes attracted by a chemotactic stimulus versus a negative control.

The difference of migration distances between stimulated and unstimulated cells became significant by 2 hr incubation (Fig. 2).

The distance of migration toward a chemotactic stimulus appeared to remain constant regardless of the diameter of the wells as long as the distance between the wells was kept at a length equal to the well diameter (Table II).

Maximum reproducibility of results was found when a third or neutral well was included. In negative controls, when the neutral well was omitted, the cells migrated slightly farther in a direction opposite the well containing non-chemotactic material.

Discussion. The agarose method for *in vitro* studies on chemotaxis has several advantages over the Boyden chamber method (1) or its modifications (2, 6, 8, 9). Unlike the chamber methods in which the cells must be fixed and stained before they are counted, the agarose method allows direct *in situ* observation of migrating cells and the migration can be easily and rapidly assessed. Results are clear and require little or no judgment because the measurements

TABLE I. Migration of Leukocytes in an Agarose System and in the Boyden System.

Material (in chemotactic well or on attractant side of Boyden chamber)	Agarose method: Migration of cells toward chemotactic well after 3 hr incubation (mm × 10^2)	Boyden method: No. cells migrating through filter
TC-199 + FCS	22.1[a] (2.2)*	19.0 (2.2)
Escherichia coli chemotactic factor	91.3 (5.8)	70.6 (7.4)
Staphylococcus epidermidis chemotactic factor	83.2 (1.8)	93.0 (5.6)

[a] Mean from a minimum of five separate determinations.
*Standard error of mean.

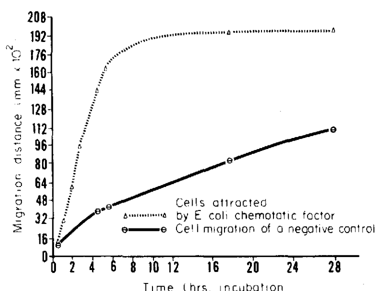

FIG. 2. Migration distances of leukocytes attracted by a chemotactic stimulus versus a negative control.

compare the distance of migration of stimulated cells with that of negative controls. Whereas chemotactic chambers may require from 50 μl or more of chemotactic material, the agarose method can be used with 5 μl or less. Also, the agarose system is less expensive and has the additional advantage of being completely disposable. This minimizes the risk of handling infectious material since most chemotactic chambers are made of plastic (e.g., Neuroprobe) and can not be autoclaved. Another advantage is that, by appropriate geometrical arrangement, several test substances may be assayed for chemotactic activity in a single plate, or conversely, several different suspensions of PMN may be examined against a single well containing a known chemotactic substance.

Because antibiotics may be used in the agarose medium and the incubation time can be less than 3 hr, the time needed to prepare an agarose system may be greatly reduced by merely dissolving the agarose in boiling water rather than autoclaving and then adding the appropriate amount of concentrated tissue culture medium. When making the wells, a plate of aluminum alloy with appropriately spaced holes may serve as a simple template for positioning the cutting tool.

TABLE II. Effect of Size of Wells and Distances Between Wells on the Migration of Leukocytes in the Agarose Method.

Diameter of well (mm)	Distance between wells (mm)	Time of incubation (hr)	Distance of migration (mm × 10²)	
			Stimulated[a] cells	Negative control
5.5	5.5	3	91.3[b] (9.2)*	22.6 (3.5)
4.0	4.0	3	87.6 (5.1)	26.9 (1.8)
2.4	2.4	3	87.5 (5.7)	20.8 (1.2)
2.4	2.4	2.5	80.5 (7.4)	
2.4	4.8	2.5	51.5 (1.7)	11.2 (2.8)
2.4	7.2	2.5	17.1 (3.5)	

[a] *Escherichia coli* chemotactic factor.
[b] Mean of at least three separate determinations.
*Standard error of mean.

Since the migrating cells can be observed directly throughout the incubation period, the agarose method may allow for kinetic studies on PMN-chemotaxin interactions. It was observed that stimulated cells had a remarkable initial rate of migration followed by an equally remarkable rate of decline. A possible explanation for this behavior may be that the PMN at first become extremely excited when the wave of chemotactic material arrives and move in a direction against the concentration gradient, thus accounting for unidirectional movement. As the concentration gradient diminishes with time, becoming equilibrated throughout the gel, the cells rapidly lose motility and assume a more random movement. Several other explanations may account for these observations and clearly more studies are indicated.

The implications of the usefulness of the agarose method as a clinical tool are, however, obvious. The actual adaptability is being investigated.

Summary. A new method for the study of *in vitro* chemotaxis has been described. It is based on the phenomenon of leukocyte migration on the surface of plastic. The method allows for direct observation of migrating cells and has several other advantages over the conventional Boyden procedure. The technique should be adaptable for use as a clinical tool.

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