

In Vitro Chemotaxis of Mouse Bone Marrow Neutrophils (40164)

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Mice are extensively used in studies of host resistance to infection but few reports deal with *in vitro* chemotactic responses of mouse polymorphonuclear leukocytes (PMNs) (1). This may be due to the relatively low yield of blood PMNs in this species (2). We show here that unfractionated marrow cell suspensions provide adequate numbers of mouse neutrophils for studies of spontaneous and chemotactic movement under agarose (3-7). In this procedure, leukocytes are pipetted into wells punched in agarose plates and migrate under the gel and over the substrate towards a chemotactic source. An elliptical cell pattern is thus generated. In the absence of a chemotactic gradient, the cells are distributed within a circle (spontaneous migration). Chemotaxis is assessed by measuring the distances covered by the cells moving towards the chemotactic source and subtracting the values for spontaneous migration (4-7). In the present experiments, counts of neutrophils that migrate towards or away from the attractant wells were found to provide a sensitive estimate of movement and to best reflect the behavior of the total migrating cell population.

Materials and methods. Animals. Swiss Webster (Taconic Farms) and AKR (Jackson Laboratories) female mice, about 20 g body weight were used.

Media and chemicals. Agarose was obtained from Calbiochem; RPMI 1640 from Gibco; bovine plasma albumin and zymosan from Sigma Chemical Co.; *S. abortus equi* lipopolysaccharide (LPS) from Difco. Calf serum was obtained from Flow Laboratories and heat inactivated.

Chemotactic sera. Sera were activated at 37° with 1 mg/ml LPS or 5 mg/ml zymosan for 60 and 30 min respectively and then incubated at 56° for 30 min. Zymosan was removed by centrifugation. Control sera were heat treated at 56° for 30 min. prior to activation. As previously shown (5), fresh mouse serum was chemotactic in this assay system,

presumably due to complement activation by the agarose.

Preparation of the agarose plates. Different batches of agarose were assayed to select the one that permitted the best migration. The method used was essentially that described in Ref. 4. Plates containing 1% agarose in RPMI 1640 with 200 mg/% NaHCO₃ were stored overnight at 4° in a humidified box before the gels were punched in the cold with a stainless steel vacuum punch (LKB, 2.4 mm).

Bone marrow cells. Animals were sacrificed and femurs and tibiae flushed with RPMI medium through a needle inserted into the medullary cavity. About 2×10^7 nucleated cells were recovered per mouse and the suspensions, which contained about 20% neutrophils were centrifuged and resuspended in RPMI at 2×10^7 cells/ml. Over a hundred wells could be loaded with the marrow from a single mouse. Adequate enrichment methods for rodent marrow neutrophils are not yet available to assess the effect of other marrow cell types on the migration of PMNs.

Migration assays. Chemotactic serum (5 μ l) was delivered into the appropriate wells by means of an Eppendorf pipette (Brinkman Instruments) 30-60 min prior to the addition of 5 μ l of cell suspensions (about 10^5 cells) to the cell wells. Plates were incubated for 1 to 4 hrs at 37° C in 5% CO₂ in air. Cells were fixed with 2% glutaraldehyde in phosphate buffered saline for 30 min, the agarose was removed, and the plates stained with 0.5% toluidine blue.

In some experiments, total numbers of migrated cells were counted on the plates with a microscope at 100 \times magnification, or on 50 \times photographic enlargements of the wells. Alternatively, cells were counted with the help of an ocular grid in two strips of 0.5 mm by 1.0 mm, one on each side of the cell well. The strips were positioned lengthwise along a line connecting the centers of the chemotactic source and the cell wells. It was possible, under the microscope, to identify the

boundaries of the cell wells and to unambiguously score the more flattened neutrophils located outside of the well boundaries. "Chemotactic differences" were obtained by subtracting cell counts in the strip farthest from the chemotactic factor well (area B Fig. 1 b insert) from counts in the strip next to the chemotactic source (area A in Fig. 1b insert). An analysis of triplicates of chemotactic differences gave a variation coefficient of 17%. Ninety-five percent of the averages of triplicates should thus be distributed within plus or minus 20% of the means.

Results. Neutrophilic granulocytes were the only marrow cells to leave the wells during the first 4 hr of incubation. Upon inspection, the migrating population consisted of mature neutrophils, with ring or polymorphic nuclei (8), equally distributed throughout the migration area. Mononuclear phagocytes moved out after 4 hr and accumulated in the inner region of the migration area.

Effect of the input of bone marrow cells and time. Total cell migration was linearly related to cell numbers pipetted into the wells, and in the presence of a chemotactic stimulus more cells left the wells at all cell inputs (Fig. 1,a). Chemotactic differences, obtained in the same experiment were also linearly related to the cell input (Fig. 1,b). These differences

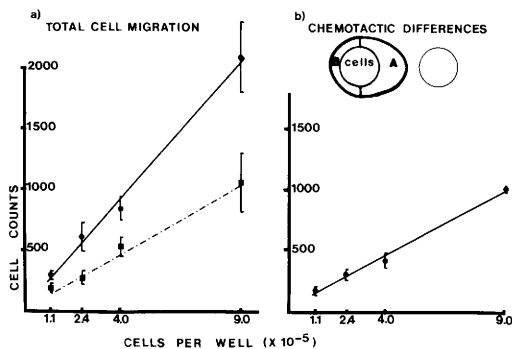


FIG. 1. Cell migration as a function of marrow cell input. Abcissae: cell numbers $\times 10^{-5}$. Ordinates: total migrated neutrophils counted on photographic enlargements. (a) Total cell migration. Solid curve: migration in response to a gradient of LPS-activated mouse serum. Broken curve: spontaneous migration. (b) Chemotactic differences obtained by subtracting counts in area B from counts in area A (see insert). Each point is an average of three or four wells. Lines were obtained by the least squares method. Vertical bars represent SE. Medium: RPMI with 10% calf serum. Time: 2 h.

were derived (in this experiment) from counts of all cells in area A minus counts in area B (see insert in Fig. 1,b). In contrast, as previously reported by others (6), the areas of migration, determined by tracing, cutting and weighing photographic enlargements, were far less sensitive to marrow cell input (data not shown). Total cell migration and chemotactic differences increased linearly between 1 and 4 hr in the presence of 10% calf serum. However, in the absence of serum the rate of cell migration declined after 2 hr.

Strip counts for chemotactic assays. Representative chemotactic differences could be obtained from cell counts in defined samples of the migration area (see Materials and Methods). Figure 2 shows that chemotactic differences obtained from these strip counts correlated adequately with the chemotactic differences derived from total counts on photographic prints of the same wells. The correlation coefficient obtained was 0.94, *df* 21, $P < 0.001$.

Medium requirements. As previously reported for human leukocytes (5) addition of serum or of serum albumin to the agarose, increased the numbers, distances and areas of chemotactic migration of the mouse granulocytes (Fig. 3). However, the chemotactic differences were similar when serum or albumin-containing media were compared in the same experiments (Fig. 4). In addition,

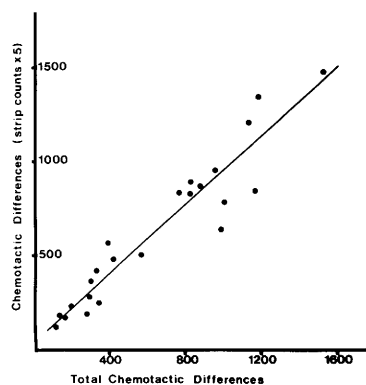


FIG. 2. Correlation between chemotactic differences estimated from total counts on enlargements and from microscope counts in strips of the migration areas. Abcissa: total chemotactic differences (enlargements). Ordinate: chemotactic differences from counts in two strips of the migration area (see text and Fig. 1). Counts multiplied by five. Points represent individual chemotactic migration wells.

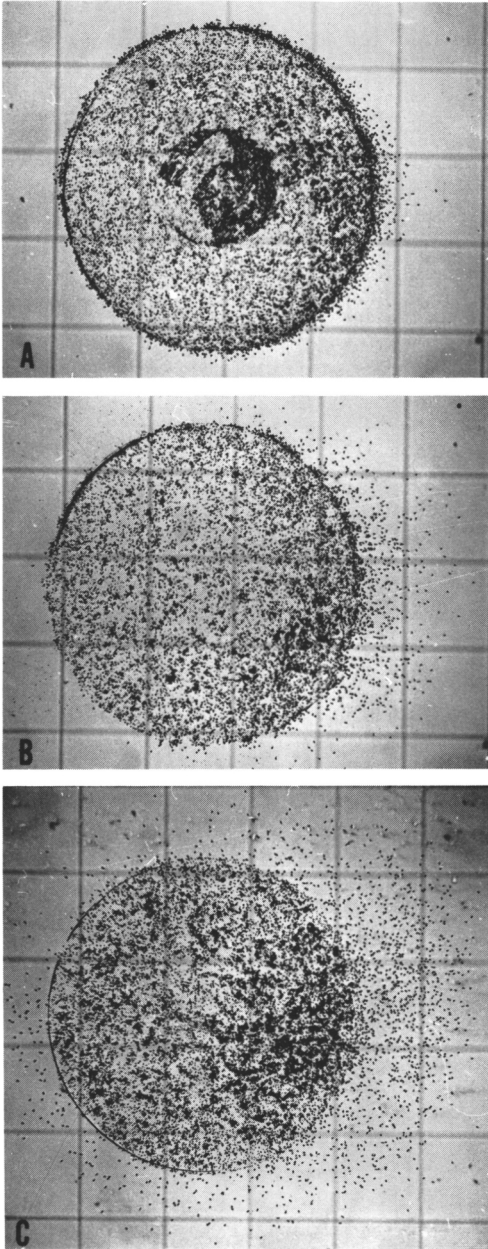


FIG. 3. Chemotactic migration in different media towards LPS serum. (A) medium RPMI alone. (B) RPMI with 2 mg/ml BSA. (c) RPMI with 10% calf serum. Migration time: 2 h. Individual migrated neutrophils are seen as fine specks outside of the wells. Each square is 660 μ M.

heat inactivated calf serum markedly increased the spontaneous migration of the PMNs.

Experiments with chemotactic sera. Figure

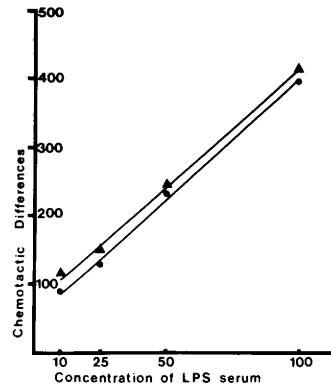


FIG. 4. Chemotactic differences as a function of the dilution of the chemotactic serum. Abcissa: percent LPS activated serum. Ordinate: chemotactic differences obtained through microscope counts of two 0.5 mm² strips of the migration area (see text). Time: 2 hr. Triangles: RPMI with 10% calf serum. Circles: RPMI with 5 mg/ml bovine albumin. The values for 100% serum are averages of five determinations each; the remaining are averages of duplicates. Curves were drawn by the least squares method.

4 shows that chemotactic differences increased linearly when the concentrations of LPS-activated mouse serum varied between 10 and 100%. Mouse neutrophils responded chemotactically to mouse, rat, rabbit or guinea pig sera activated with either LPS or zymosan. Control, heat inactivated sera, to which LPS or zymosan were added were not chemotactic. Activated human serum was not chemotactic for the mouse cells, whereas the same serum in a parallel experiment was active for human PMNs. The chemotactic factor present in activated mouse serum was presumably C5a since zymosan-treated C5-deficient AKR serum was not chemotactic. Heat inactivated calf serum added to the attractant well was only minimally chemotactic at full strength.

Discussion. Bone marrow provides adequate numbers of mouse granulocytes for spontaneous and chemotactic migration under agarose. Mouse marrow as a source of neutrophils may be preferable to exudate leukocytes for chemotaxis studies, since exudate cells have already been exposed to chemotactic factors and may be partially deactivated (Ref. 10, p. 47). Because separation of the neutrophils and lysis of erythrocytes are not required, the cells can be placed in the wells soon after collection and are subjected to only

a minimum of experimental stress. The cell count assay permits the use of small numbers of cells per well (about 10^5 nucleated cells, containing 2×10^4 neutrophils) and short migration periods, conditions that do not allow precise estimates of migration distances or areas. Furthermore, cell counts best reflect the behavior of total migrating populations rather than that of a few leading cells.

The agarose assay offers several advantages over the filter methods (9–12) for studies of the movement of PMNs and other cells. (a) The behavior of the living cells can be observed and filmed (13). (b) The nature of the substrate can be modified to provide information on cell-substrate interactions involved in cell movement, and, in particular, to clarify the relationship between cell adhesion and cell movement (14). (c) The assay may permit an easier distinction between agents that affect chemokinesis (increased or decreased spontaneous migration) or chemotaxis (directional migration) than the filter assays (15). (d) The method described may also be useful in studies of murine leukocyte migration inhibitory factors (3). Finally, the proposed procedure may permit easy screening of mouse strains to define the genetics of the chemotactic response in the mouse (16).

Summary. Unfractionated marrow cell suspensions provide adequate numbers of mouse neutrophils for studies of spontaneous and chemotactic migration under agarose. Mature neutrophils were the only cells to leave the wells in the first 4 hr. For chemotactic studies, quantitation involved microscope counts of cells in selected samples of the migration areas, and the chemotactic responses were linearly related to the dilution of activated

serum. Mouse, rat, guinea pig or rabbit activated sera but not human sera were chemotactic for mouse granulocytes. Spontaneous migration was determined by total counts of neutrophils leaving the wells.

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