Integrating Conflicting Chemotactic Signals: The Role of Memory in Leukocyte Navigation

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Abstract. Leukocytes navigate through complex chemoattractant arrays, and in so doing, they must migrate from one chemoattractant source to another. By evaluating directional persistence and chemotaxis during neutrophil migration under agarose, we show that cells migrating away from a local chemoattractant, against a gradient, display true chemotaxis to distant agonists, often behaving as if the local gradient were without effect. We describe two interrelated properties of migrating cells that allow this to occur. First, migrating leukocytes can integrate competing chemoattractant signals, responding as if to the vector sum of the orienting signals present. Second, migrating cells display memory of their recent environment: cells' perception of the relative strength of orienting signals is influenced by their history, so that cells prioritize newly arising or newly encountered attractants. We propose that this cellular memory, by promoting sequential chemotaxis to one attractant after another, is in fact responsible for the integration of competitive orienting signals over time, and allows combinations of chemoattractants to guide leukocytes in a step-by-step fashion to their destinations within tissues.

Key words: leukocyte • neutrophil • chemokinesis • chemotaxis • cellular memory

EXQUISITELY choreographed cell movements underlie many fundamental biological processes. In embryological development, cells migrate together to form complex organs, and neurons follow tortuous courses to their destinations. Similarly, immunologic surveillance requires coordinated, highly specific cell movements; to combat invading pathogens, neutrophils and monocytes must migrate to specific sites within infected tissues, and to respond to foreign antigens, lymphocytes must circulate through the body visiting specific functional zones within lymphoid organs. The mechanisms that direct and control cell movements with such specificity are just beginning to be elucidated.

A diverse group of chemoattractant molecules are thought to play a critical role in directing leukocyte homing within tissues. For many years, several classical chemoattractants produced in inflamed tissues, such as bacterial peptides, have been known to elicit neutrophil migration. In recent years, a multitude of related protein chemoattractants for leukocytes, called chemokines, have been characterized (for reviews see Baggiolini et al., 1997; Luster, 1998; Zlotnik et al., 1999). Both chemokines and classical attractants bind to G protein–coupled receptors on leukocyte surfaces, and elicit cell orientation and directional migration when presented in a concentration gradient. Mice deficient in the ability to produce or respond to chemokines or classical attractants can display marked defects in leukocyte localization to and within their recruiting tissues (Cook et al., 1995; Förster et al., 1996; Gao et al., 1997; Hopken et al., 1997; Kurihara et al., 1997).

The characterization of a wide variety of leukocyte attractants and their receptors has painted a complex picture of the migratory environment within leukocyte-recruiting tissues. First, studies have shown that each leukocyte type can respond to multiple different chemoattractants (Baggiolini et al., 1997; Luster, 1998; Zlotnik et al., 1999). Furthermore, in general, a diverse array of chemoattractants is produced by different cell types within leukocyte-recruiting tissues (Becker et al., 1994; Schroder, 1995; Wenzel and Abboud, 1995; Gillitzer et al., 1996; Glabinski et al., 1996; Gonzalo et al., 1996; Spanaus et al., 1997; Luster, 1998). Therefore, a leukocyte entering a recruiting tissue likely encounters many different chemotactic signals to which it can respond. In a recent study, we showed that in such settings, neutrophils can navigate to their target by

Portions of this work are included, along with additional experiments, in Dr. Foxman's Ph.D. dissertation (Foxman, 1999).

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migrating in sequence toward one chemoattractant source and then another (Foxman et al., 1997). This behavior permits combinations of chemoattractants to act in series to guide cells to specific destinations. To find their way through such a complex chemoattractant environment, cells require mechanisms to integrate and prioritize the signals they receive.

Some mechanisms whereby leukocytes prioritize chemotactic signals are known. For example, neutrophils can prioritize signals from their phagocytic targets over more general recruitment signals (Foxman et al., 1997), likely via heterologous receptor desensitization by dominant agonists (Wilde et al., 1989; Dobos et al., 1992; Tomhave et al., 1994; Richardson et al., 1995; Campbell et al., 1997; Foxman et al., 1997; Kitayama et al., 1997; Sabroe et al., 1997). This ability is expected to allow neutrophils to find their end targets efficiently, even in settings in which regulatory chemoattractant gradients are also present (Campbell, 1997; Foxman et al., 1997). However, of the many leukocyte chemoattractants that have been described, dominant agonists appear to be exceedingly outnumbered by chemoattractants, such as the chemokines, that do not suppress cellular responses to other attractants. When a leukocyte encounters overlapping arrays of these nondominant, generally regulatory cell–derived agonists, the chemoattractant receptors involved do not engage in cross-desensitization, but rather signal simultaneously within the cell. However, even in the presence of such nondominant agonists, cells must be able to prioritize chemotactic signals so that they can successfully navigate through chemoattractant arrays. In particular, to respond in series to sequentially encountered gradients, leukocytes must be able to migrate away from one chemoattractant source towards another, as indeed they can (Foxman et al., 1997; Kitayama, 1997).

To learn more about how leukocytes process conflicting chemotactic signals, we studied neutrophil orientation in the under agarose assay (Foxman et al., 1997) evaluating the effects of directional persistence versus chemotaxis on cell behavior in the presence of opposing gradients of two attractants: the chemokine interleukin 8 (IL-8)[1] and the classical lipid attractant leukotriene B4 (LTB4). These agonists are both produced by regulatory cells in inflamed tissues, and neither suppresses cellular responses to the other (Foxman et al., 1997). We demonstrate that cells migrating away from a local chemoattractant source actually chemotax towards distant attractants. However, neutrophils can clearly experience and respond as if to the vector sum of orienting signals from competing chemoattractants, resulting in migration directed between the two agonist sources (when the two independent attractants are presented equidistant at an angle). Reconciling these observations, we demonstrate that a cell's chemotactic bias in a given chemoattractant array depends not only on the gradients present, but also on the cell's memory of its recent chemoattractant environment. We propose a model in which a delay in adaptation constitutes this cellular memory and, by allowing cells to prioritize newly arising or distant attractants, leads to the integration of conflicting chemotactic signals over time and the step-by-step navigation of leukocytes in response to sequentially encountered chemoattractant sources.

1. *Abbreviations used in this paper:* FMI, forward migration index; IL-8, interleukin 8; LTB4, leukotriene B4; r, correlation coefficient.

Materials and Methods

Under Agarose Assay

Assay Procedures. The procedures of Nelson et al. (1975) were adapted for our experiments, as described previously (Foxman et al., 1997), with modifications as outlined in Results. Neutrophils were isolated using standard techniques (see Foxman et al., 1997). For each cell tracking experiment, assays were set up and allowed to incubate at 37°C for 75 min, at which time they were moved to a warmed (37°C) microscope stage, and cell movements were recorded for 15 min.

Agarose Gel. Agarose gels contained endotoxin-tested RPMI-1640 (Sigma Chemical Co.), 10% heat-inactivated bovine calf serum (serum inactivated at 57°C for 25 min), 1.2% agarose (GIBCO BRL), and 10 mM Hepes (GIBCO BRL), pH 7.2. For standard assays, five 3.5-mm-diam holes were cut 2- or 1.5-mm apart in a linear array. For experiments with three equidistant wells in an equilateral triangle, a template-guided steel punch was used to form three 3-mm-diam wells 3.3-mm apart.

Migration Medium. Cells and agonists were suspended in RPMI-1640, 10% heat-inactivated bovine calf serum, and 10 mM Hepes, pH 7.2.

Chemoattractants. Chemoattractants used were Leukotriene B_4 (Sigma Chemical Co.) and recombinant human interleukin-8 (a gift from Antal Rot, Sandoz-Forschungsinstitut, Vienna, Austria).

Video and Confocal Microscopy. Images were captured every 30 s on an inverted microscope (captured area = 780 μm orthogonal to the axis of the wells × 584 μm along axis of wells) or every 15 s on a laser scanning confocal microscope (MRC 1024; Bio-Rad Laboratories, Inc.) using Lasersharp software version 2.1 (Bio-Rad; captured area = 800 × 800 μm). For confocal microscopy experiments, neutrophils were labeled with 100 nM (final concentration/10^7 cells) of either SYTO-13 (green) or SYTO-15 (orange; Molecular Probes Inc.) for 25 min at room temperature in migration medium, washed, resuspended, and stored on ice until the start of the assay.

Uniform Field of LTB4. To create a uniform field of chemoattractant, 1 nM LTB4 was incorporated into the agarose gel. Two wells, 7.5 mm apart, were cut in the gel. At t = 0, each well was filled with 10^5 cells in 20 μl of medium containing 1 nM LTB4. At t = 60 min (15 min before filming), well contents were overlaid with agarose containing 1 nM LTB4.

Data Analysis

Cell Tracking. Each videotape (or series of confocal images) was converted to an NIH Image time-lapse movie, with one image every 30 (or 15) s. Cells moved on average 1–1.5 cell lengths/min, so cells moved less than a cell length between frames and could be identified from one image to the next. (The length of migrating cells was ~20 μm as cells spread out significantly under the agarose.) An analysis region was defined based on the position of the filmed cells relative to the chemoattractant-containing wells, and only cells that were the desired distance from each well at the beginning of the movie were tracked. The analysis region always excluded cells within 100 μm of the field edge because they could not be reliably tracked for the entire 11.5-min analysis period. Usually, 90% of the cells within the analysis region could be followed for the entire 11.5-min analysis period (always = 87.5%). Once the analysis region was defined, each cell was numbered and its x, y coordinates were measured on the first image and on every subsequent image in the image stack, with the x-axis parallel to the edge of the cell starting well and the y-axis orthogonal to the well edge (parallel to a line connecting the starting well and the distant well). The (x, y) data for each cell was exported to a Microsoft EXCEL spreadsheet and used for the subsequent calculations of chemotactic bias.

The filmed field was aligned so that the axis of the experimental wells on the plate passed vertically through the center of the field, with the forward direction defined as directly away from the cell starting well.

Determination of the Decay of Cellular Persistence. For cells migrating in a uniform field, the direction of a cell's motion during a given time interval was calculated trigonometrically using the cell's (x, y) coordinates. To determine an average direction at each time point, the cell's angle relative to the forward direction (0°) was calculated over three overlapping time intervals; for example, the cell's initial direction was calculated as the average of the cell's angle from 0 to 1 min, 0.5 to 1.5 min, and 1 to 2 min of

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observation. A cell was considered to be persistent until it achieved an angle >90° from its starting angle. To assure accurate assessment of the starting angle, the few cells that were not traveling at least 1 cell length/min (20 μm/min) during the initial 2-min period were excluded from the analysis. The analysis was begun within 3 min of the start of the videotape, at the first time at which the cell was traveling at least 20 μm/min.

Determination of Chemotactic Bias. Each cell's (x, y) coordinates were used to calculate the cell's efficiency of forward migration, or forward migration index (FMI), during an initial 2-min analysis period, and its efficiency of forward migration during a subsequent test period of 4–9.5 min (see Results). The FMI was calculated as follows:

$$\text{FMI} = \frac{\text{Forward Progress}}{\text{Total Path Length}} = \frac{\Sigma\Delta y}{\Sigma((\Delta x^2 + \Delta y^2)^{-1/2})} \quad (1)$$

with Δx and Δy assessed for each 30-s interval throughout the analysis period. For most experiments, the FMI was only calculated for the initial 2 min of observation and the subsequent 9.5 min; 11.5 min was the longest period for which nearly all cells selected could be followed without interruption (without leaving the field), and thus, an initial 2-min and subsequent 9.5-min observation provides the most reliable measure of the cells' time-averaged behavior. For each experimental condition, we plotted the initial versus subsequent FMI for all tracked cells and fitted a line through these data points by linear regression. The y-intercept of this line indicates the cells' chemotactic bias (see Results).

Statistical Methods. We used standard statistical techniques to weigh and pool data acquired on different days. Intercepts of regression lines were compared using a *t* test.

Results

Effect of Directional Persistence on Neutrophil Migration

To understand the behavior of neutrophils migrating in overlapping chemoattractant gradients, we needed to develop a quantitative method to evaluate chemotaxis (directed cell motility) and to distinguish its contribution from the other factors influencing cell migration in our model. We first characterized cell migration in the presence of a uniform field of chemoattractant: chemoattractants stimulate random cell motility (chemokinesis) when present at a uniform concentration (Zigmond and Hirsch, 1973; Zigmond et al., 1981). We incorporated LTB4 directly into an agarose gel, at an optimal concentration for stimulation of cell movement (1 nM). Neutrophils (in medium containing the same concentration of LTB4) were added to a well in the gel and allowed to migrate from their starting position for 75 min, videotaped, and analyzed over the next 15-min period.

To assess the initial orientation of the cells within the field of view, we determined the average angle of cell migration during the first 2 min of the 15-min observation period. As shown in Fig. 1 a, the distribution of cell orientations was not random, but rather a majority of videotaped cells pointed away from the starting well, or forward (27/43 or 63%), in spite of the absence of a gradient. We postulated that this initial forward orientation might be the result of the following: (a) a mass action effect, in which most cells enter the field of observation from the direction of the starting well coupled with (b) a directional persistence, the well-described tendency of neutrophils to continue in their recent direction, rarely making sharp turns, even in the absence of an orienting gradient (Allan and Wilkinson, 1978; Zigmond et al., 1981; Shields and Hatson, 1985; Burton et al., 1987). Together, these two phenomena could result in an initial forward bias in cell orientation. The forward bias could also result from real chemotaxis because of, for example, cellular chemoattractant destruction. To distinguish between these possibilities, we analyzed cell behavior in more detail.

Consistent with previous reports, cells migrating in a uniform field in our assay displayed considerable directional persistence: after 5 min, only ~50% of cells had turned >90° from their starting direction; by 10 min, ~90% of cells had turned (Fig. 1 b). To the degree that cells persist in their initial directions, an initial forward bias in cell orientation would be expected to lead to a forward bias in subsequent cell movements. Therefore, any analysis of the cells' chemotactic behavior must be corrected for the cells' initial orientation. To assess the relationship between the initial and subsequent direction of cell movements, we calculated each cell's FMI during an initial 2-min observation period, and then during subsequent 4, 7, or 9.5-min observation periods. The FMI is the ratio of the net distance the cell progressed in the forward direction (away from the starting well) to the total distance the cell traveled as it wandered through the videotaped field (see Materials and Methods). The FMI measures the efficiency of a cell's forward migration during a given time interval. (In experiments involving migration to a distant attractant source, the forward migration index is equivalent to the McCutcheon index used by previous investigators to compare a cell's most direct path to a gradient source to its total path length [McCutcheon, 1946; Zigmond, 1974]).

Fig. 1, c–e, show the relationship between the initial and subsequent forward migration indices for cells migrating in a uniform field of LTB4. Fig. 1 c shows the subsequent FMI, calculated over a 4-min period (from t = 2–6 min), plotted against the initial index, calculated over the first 2-min period of observation (t = 0–2 min). The regression line through these data has a positive slope (+0.55), indicating that cells that start out moving forward tend to continue to progress forward during the subsequent 4-min period, and that cells that initially move backward tend to continue to progress backward. The correlation between the initial and subsequent FMI is statistically significant (correlation coefficient r = 0.58, significantly different from zero with $P < 0.0001$). The slope of the linear regression line of the initial versus subsequent FMI is an indicator of the degree to which cells persist in their initial direction; therefore, it can be considered a persistence index. Fig. 1, d and e, plots the cells' forward migration indices, as calculated over longer intervals (of 7- and 9.5-min duration), against their initial migration indices determined in the first 2 min of observation. Again, there is a significant positive correlation between the cells' initial and subsequent movements, but it becomes less prominent as cells are tracked for longer intervals (when the subsequent migration index is determined over 7 min, from t = 2–9 min, the slope = 0.33, and r = 0.41, $P = 0.01$; when determined over a 9.5-min period, from t = 2–11.5 min, the slope = 0.32, and r = 0.42). The correlation between the initial and subsequent forward migration indices, and the reduction of this correlation over time, is consistent with the effect of directional persistence on cell movement, and with the decay of this effect as cells are followed for a longer time interval. Cells displayed similar behavior when migrating

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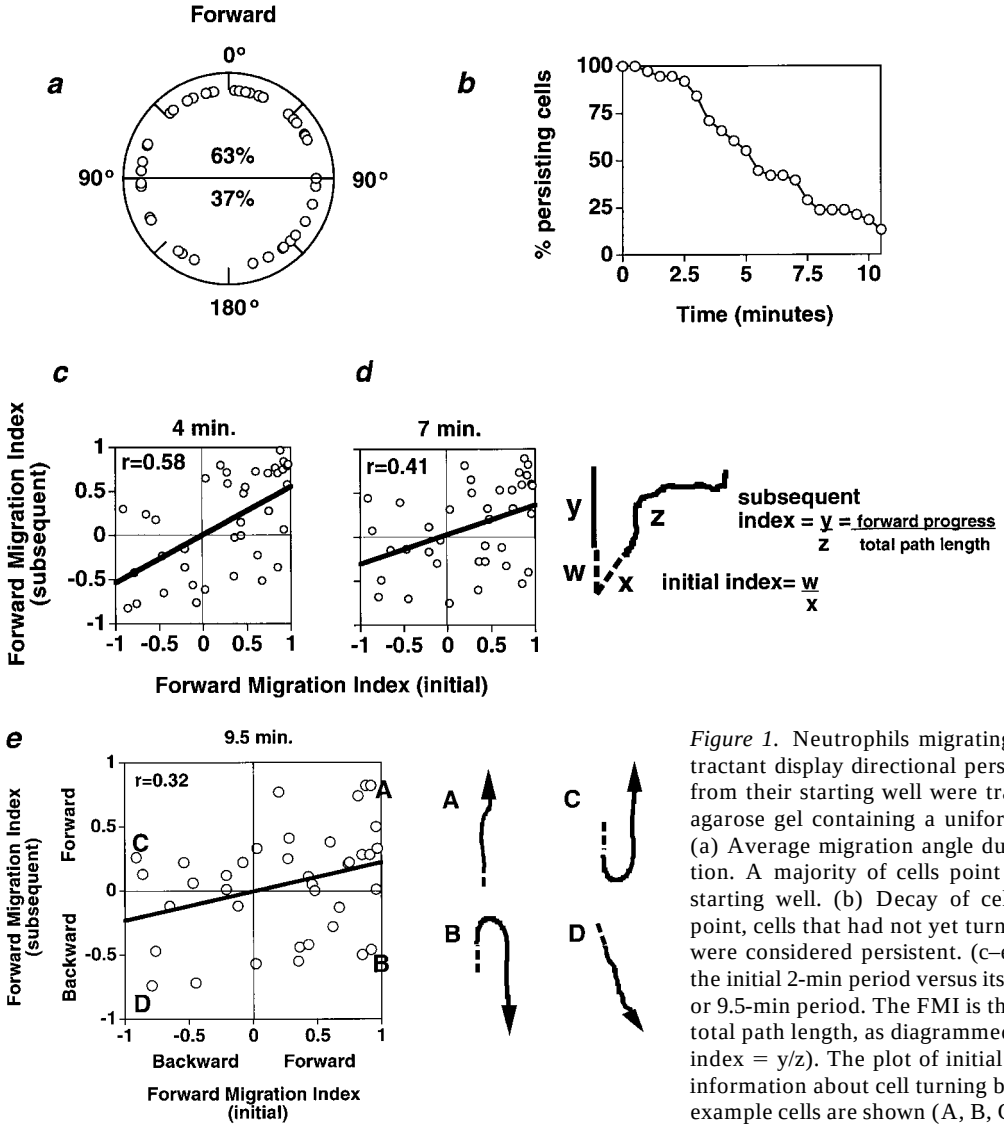

Figure 1. Neutrophils migrating in a uniform field of chemoattractant display directional persistence. Neutrophils 400–700 μm from their starting well were tracked as they migrated under an agarose gel containing a uniform concentration of LTB4 (a–d). (a) Average migration angle during the initial 2 min of observation. A majority of cells point forward (0°), or away from the starting well. (b) Decay of cellular persistence. At each time point, cells that had not yet turned >90° from their starting angle were considered persistent. (c–e) Plot of each cell's FMI during the initial 2-min period versus its FMI during the subsequent 4-, 7-, or 9.5-min period. The FMI is the ratio of net forward progress to total path length, as diagrammed (initial index = w/x; subsequent index = y/z). The plot of initial versus subsequent FMI provides information about cell turning behavior. Possible paths of several example cells are shown (A, B, C, and D), as discussed in the text.

in a uniform field containing both IL-8 and LTB4 (not shown).

The plots comparing the cells' initial and subsequent movement (Fig. 1) describe, in essence, neutrophils' turning behavior. To understand the data presented in Fig. 1 e more fully, it is useful to consider several example cells. Cell A pointed forward during the initial 2-min analysis period, as indicated by its positive initial FMI (+0.92), and it continued to progress forward during the subsequent 9.5-min analysis period. Cell B, like A, pointed forward during the initial 2-min analysis period, but did not maintain this course throughout the subsequent period. Cell B has a negative FMI during the 9.5-min tracking period (−0.46), indicating that, overall, this cell went backward during that time, and therefore, must have turned from its initial direction. Cell C pointed backward during the initial period, but turned during the subsequent period because, overall, it progressed forward during the subsequent 9.5-min analysis period (subsequent FMI = +0.26), whereas cell D also initially pointed backward, as evidenced by its negative initial forward migration index (−0.79), and it continued to progress backward during the subsequent period.

Once the relationship between the cells' starting directions and their subsequent progress is known, it can be used to assess whether cells display a directional turning bias. To do so, we asked to what degree an average cell progresses forward if it starts out pointing neither forward nor backward, that is, pointing to the left or right, with an initial FMI of zero. This value is equivalent to the y-intercept of the regression line that fits the initial versus subsequent migration index data. Note that the y-intercept of the plot for cells migrating in a uniform field of chemoattractant, in our system, is always close to zero (Fig. 1, c–e), so that a cell starting out with no bias forward or backward, on average, makes no net progress forward or backward. In fact, this is the behavior expected for cells migrating in the absence of a chemotactic gradient. Thus, by comparing cells' initial direction to their subsequent movement, we have arrived at a measurement of directed cell movement normalized for cells' starting orientations. This measurement, the y-intercept, describes the turning

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bias associated with true chemotaxis and is hereafter termed the chemotactic bias. The regression line through the cells' initial versus subsequent forward migration indices is therefore: subsequent FMI = chemotactic bias + (persistence index) × (initial FMI).

Measurement of Chemotactic Bias in a Gradient

Next, we assessed the behavior of cells migrating in an LTB4 gradient. In this experiment, we videotaped cells migrating from their starting well towards an LTB4-containing well 2 mm away. The initial and subsequent migration indices were determined for each cell as above. In this and the following experiments, the subsequent FMI was determined over a 9.5-min period only. The plot comparing initial and subsequent movement reveals several noteworthy features of the cells' behavior (Fig. 2). First, like cells migrating in a uniform field, cells that start out migrating forward tend to progress forward during the subsequent analysis period (i.e., then tend to have positive subsequent forward migration indices). However, in contrast to cells migrating in a uniform field, even cells that start out pointing backward, or towards the cell starting well, tend to make net progress forward (up the LTB4 gradient) during the subsequent 9.5 min. Consequently, the y-intercept of the regression line through these data, or the chemotactic bias, is positive (0.34, with a 95% confidence interval of 0.24–0.43). This indicates that, in this setting, an average cell that started out pointing orthogonal to the LTB4 gradient makes net progress forward, toward the LTB4 source, during the subsequent 9.5 min. Based on these data, we can conclude that cells exposed to this LTB4 gradient display a significant turning bias, and tend to progress towards the gradient source, independent of initial orientation and of directional persistence. The minimal residual effect of directional persistence is apparent in the slight positive slope of the regression line through these data.

The experiments that follow were analyzed in the same way, and in each case the y-intercept of the regression line relating the initial to subsequent forward migration indices is presented as the population's chemotactic bias.

Neutrophils Migrating from a Local Chemoattractant Source Can Orient and Chemotax Towards a Distant Chemoattractant

We were initially interested in understanding the ability of neutrophils to migrate away from one chemoattractant source in response to another, more distant agonist (Foxman et al., 1997). To this end, we tracked neutrophils migrating from a well containing IL-8 towards a well containing LTB4 (and vice versa). For the chemoattractant sources, we selected a concentration of LTB4 and IL-8 each eliciting optimal neutrophil migration from a distant well. At these concentrations, cells migrate the same distance toward a distant IL-8 source in the presence and absence of a local LTB4 source (and vice versa) (Foxman et al., 1997). This behavior is observed in spite of the fact that quantitative measurements of chemoattractant concentrations indicate that the mean concentration and gradient slope of the local agonist should itself be able to mediate

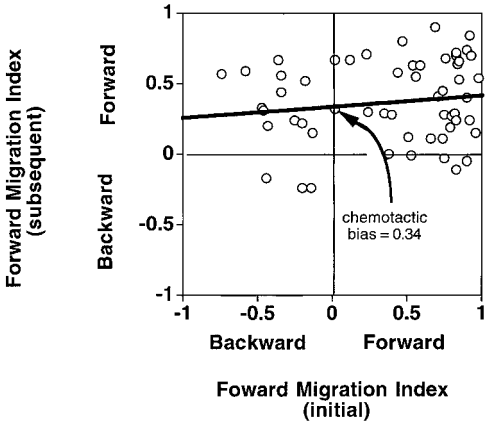

Figure 2. Neutrophils migrating in an LTB4 gradient display a chemotactic bias, independent of the effects of directional persistence. Neutrophils 400–700 μm from the starting well were tracked as they migrated under an agarose gel in response to a distant LTB4 source. The plot shows each cell's FMI over an initial 2-min period versus its FMI during the subsequent 9.5-min period. The y-intercept of the regression line is significantly greater than zero (0.34, with a 95% confidence interval of 0.24–0.43), indicating a chemotactic bias towards the LTB4 source.

chemotaxis of neutrophils in the opposite direction (Foxman et al., 1997).

We reasoned that this migration in the context of opposing or even conflicting gradients could represent either true chemotaxis or, possibly, enhanced chemokinesis in the region of chemoattractant overlap. To distinguish between these possibilities, we assessed cell migration behavior in the presence of opposing gradients as follows: cells were placed in a well with either IL-8 or LTB4 (the local well), and the other chemoattractant was added to the distant well 2 mm away. From 75 to 90 min, cells 400–700 μm from the local agonist (1,300–1,600 μm from the distant well) were filmed and their migration bias assessed, exactly as described above for the previous experiment. We found that the mean velocity of migrating cells was the same (24–27 μm/min) whether one or both chemoattractants were present, suggesting that altered motility could not explain cell behavior. Furthermore, neutrophils displayed a true chemotaxis toward a distant IL-8 source in the presence of a local LTB4 source, equivalent to that displayed in the absence of LTB4; similarly, cells chemotaxed towards a distant LTB4 source equivalently, in the presence or absence of a local IL-8 source (Fig. 3 a).

Additional control experiments were performed to determine whether IL-8 and LTB4 sources, at the concentrations used here, create functional gradients at a nearby site (400–700 μm). In these control experiments, we allowed naive neutrophils to migrate toward an IL-8 or LTB4 source from a starting well positioned closer to the chemoattractant, so that these cells would be 400–700 μm away from the source during the relevant time interval (75–90 min). Under these conditions, neutrophils exhibited significant chemotaxis to both a local IL-8 source and a local LTB4 source (Fig. 3 b), confirming that in the previous experiment (Fig. 3 a), a functional orienting gradient

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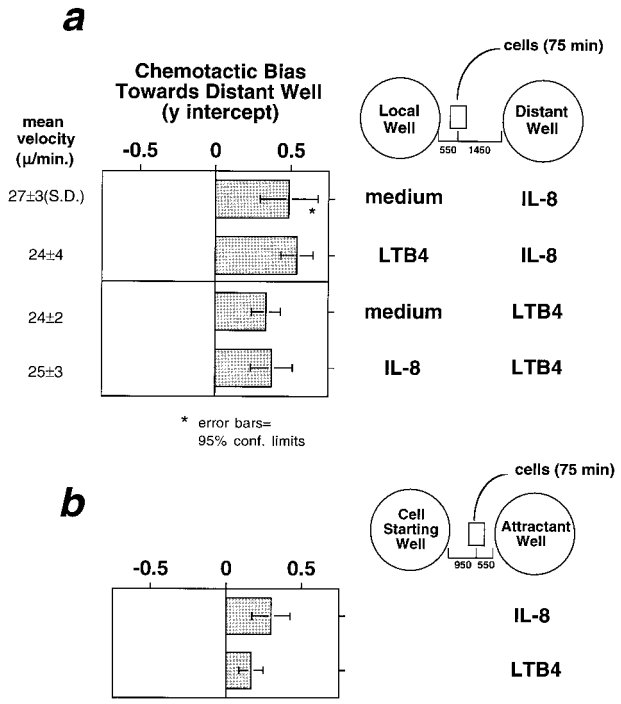

Figure 3. Neutrophils migrating from a local chemoattractant source can chemotax towards a distant chemoattractant. (a) Neutrophils were tracked as they migrated from a well containing IL-8 (1 pmol), LTB4 (0.3 pmol), or medium only towards a distant well containing IL-8 (1 pmol) or LTB4 (0.3 pmol). The bar graph shows the chemotactic bias, or the y-intercept of the initial versus subsequent FMI plot, exhibited by cells migrating under various conditions. The initial versus subsequent FMI plot for cells migrating from medium only towards LTB4 is shown in Fig. 2; the other plots are not shown. The average velocity of cells was similar under all conditions (24–27 μm/min, as indicated in the figure). We tested each condition on the same day, using neutrophils from the same blood donor, and scored 30–60 cells per condition. (b) Neutrophils were tracked as they migrated towards an IL-8 or LTB4 source 1.5 mm away. Cells 400–700 μm from the chemoattractant well at the time point relevant to the experiment described in (a) were analyzed and their chemotactic bias was determined. Bar lengths represent the chemotactic bias, or y-intercept of the initial versus subsequent FMI plot. Error bars represent the 95% confidence interval for the y-intercept. Distances from each well to the center of the videotaped field are indicated on the diagram (in micrometers).

of the local agonist exists. We conclude that, under these experimental conditions, neutrophils display true chemotaxis to a distant agonist source even when migrating down an effective gradient of a local agonist.

Neutrophils Can Integrate Directional Signals from Chemoattractant Sources Presented at an Angle

The above results suggest that neutrophils can integrate competing directional signals in such a way that they continue to migrate directionally. One possibility is that this integration, in effect, involves a vector sum of the competing orienting signals. If so, we reasoned that it should be possible to demonstrate such vectorial integration by asking neutrophils to respond to two equidistant agonist sources presented at an angle. We adjusted the under agarose assay by cutting three wells in a triangular configuration, and introducing neutrophils into one well and chemoattractants into the other two wells.

We performed a series of assays in which neutrophils and chemoattractants were added to the wells and cells were allowed to migrate for 2.5 h. Photographs of cell migration patterns are shown in Fig. 4. When only one well contained chemoattractant, neutrophils migrated towards that well only. When both wells contained the same chemoattractant, either LTB4 or IL-8, neutrophils migrated furthest in the direction of each chemoattractant well. This is the migration pattern that would be expected if each cell migrated up the steepest local chemotactic gradient encountered. (From previous gradient measurements [Foxman et al., 1997], we know that there is a trough of lower agonist concentration up the midline, and that the steepest local gradients cells would experience would, thus, be in the direction of each chemoattractant source well.)

However, notably when one well contained IL-8 and the other contained LTB4, cells migrated furthest in a direction between the two chemoattractant sources. This is the pattern that would be expected if the direction of the entire migrating population is influenced by both chemoattractants. The possibility that this pattern could result from a combined effect of the chemoattractants on cell velocity is unlikely, since cells migrate with about the same velocity in the presence of either chemoattractant as they do the presence of both together (on average 20–30 μm/min; see for example Fig. 3 a). This result confirms that most neutrophils can respond to both agonists, and suggests that the cells' integrated response to two different agonists can result in predominant cell targeting to a point between the sources, which is consistent with a model of vectorial integration of the two signals.

A Role for Cellular Memory: Neutrophils' Chemotactic Behavior Depends upon Their Previous Chemoattractant Environment

We have shown that neutrophils behave as if they can integrate directional information from competing chemotactic signals (Fig. 4). On the other hand, in the initial experiments presented in Fig. 3, we saw that neutrophils can chemotax away from a local chemoattractant source towards a distant source of a different chemoattractant, without a noticeable effect of the local agonist on the cells' chemotactic bias. Why do neutrophils appear to ignore the local gradient in this context? One possibility is that cells somehow prioritize signals from a distant gradient over those from an otherwise equivalent local agonist source: this might be possible, for example, if the strength of the perceived orienting signals (and thus the behavior of cells) is influenced by the cells' prior history.

To evaluate this possibility, we altered the conditions of the assay: we placed the IL-8 and LTB4 wells closer together (1.5 mm), and used slightly different amounts of IL-8 and LTB4 (0.5 and 0.1 pmol, respectively), so that cells coming from either direction would enter the common central region (600–900 μm from each well) at 75 min. If the cells' chemotactic bias simply reflects an integration of

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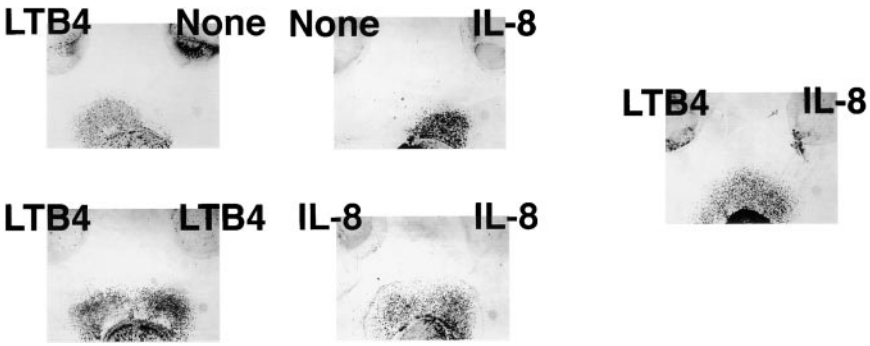

Figure 4. Neutrophils can integrate directional signals from chemoattractant sources presented at an angle. A neutrophil-containing well and two chemoattractant source wells were placed in an equilateral triangle. Chemoattractant source wells contained medium, IL-8 (10 pmol), or LTB4 (10 pmol). Cells were allowed to migrate for 150 min, after which they were fixed, stained, and photographed. In the presence of a single chemoattractant source (top left and middle), cells migrated toward that source only. In the presence of two identical chemoattractant sources (bottom left and middle), the majority of cells migrated furthest in the direction of one chemoattractant source or the other. In the presence of two different chemoattractant sources (right), cells migrated in a broad front, with the majority of cells migrating furthest in an intermediate direction between the two sources. Each condition was performed in triplicate; photographs show representative results.

the gradient vectors present, we anticipated that the cells in the middle region would behave the same way regardless of the direction from which they had migrated.

We observed that, under these conditions, cells that had migrated into the central region between the two wells always displayed true chemotaxis towards whichever agonist was presented in the distant well: neutrophils that had migrated from LTB4 into the central region displayed a significant chemotactic bias toward the initially distant IL-8 source, and vice versa. Furthermore, the chemotactic bias cells displayed towards an initially distant chemoattractant source was only slightly lower in the presence of an opposing gradient of the other chemoattractant (Fig. 5 a, top, and Table I).

One possibility is that cells originating in one chemoattractant source well alter that chemoattractant source in such a way that it no longer generates a functional gradient (i.e., by degrading the chemoattractant). To address this issue, the same experiment was performed with neutrophils starting simultaneously in both chemoattractant source wells. To distinguish cells that originated in the LTB4-containing well from cells that originated in the IL-8–containing well, cells were labeled with either a green or an orange nuclear dye and cell movements were tracked using confocal microscopy. The chemotactic biases of these distinct cells populations were evaluated as cells migrated within a central region between the two chemoattractant sources (cells 550–950 μm from each well at 75 min). Again, we found that cells that had migrated into the central region between the two wells always displayed true chemotaxis towards whichever agonist was presented in the distant well (Fig. 5 a, bottom, and Table I). By the end of the observation period, cells had intermingled significantly (Fig. 5 b). Because both cell populations were present in the central region simultaneously, we conclude that cells from different starting locations display different chemotactic behavior even when experiencing an identical chemotactic field. These results suggest that cellular chemotaxis in the presence of conflicting gradients is influenced by cellular memory, which alters the perceived strength of the orienting signals in a given chemotactic field based on the cells' previous chemoattractant environment, in such a way that cells respond preferentially to gradients of novel chemoattractants.

Discussion

Previously, we have shown that leukocytes can navigate in a step-by-step fashion through complex arrays of chemoattractants (Foxman et al., 1997). Here, we have sought to understand the mechanisms whereby leukocytes can respond sequentially to regulatory cell–derived chemoattractants. Our experiments reveal two fundamental properties of leukocyte chemotactic responses: an ability of neutrophils to integrate competing directional signals, as if responding to the vector sum of orienting gradients present, and the dependence of the resulting directional responses on the cells' prior history (i.e., on cellular memory of the recent chemoattractant environment). We propose that these interrelated properties allow leukocytes to navigate successfully through complex chemoattractant arrays. We shall discuss the results of our experiments in the context of these concepts.

Vector Integration: Leukocytes Can Integrate Orienting Signals from Distinct Chemoattractant Sources

The present studies were initiated to understand the cellular responses that allow neutrophils to migrate efficiently from one agonist source to another (Foxman et al., 1997).

Table I. Neutrophil Chemotactic Biases in Opposing Gradients

Distant well		LTB4		IL-8		IL-8		LTB4	
Starting well		none		none		LTB4		IL-8	
		y-int	S.E.	y-int	S.E.	y-int	S.E.	y-int	S.E.
Unidirectional	1	0.25	0.05	0.25	0.08	0.17	0.06	0.19	0.06
	2	0.20	0.05	0.24	0.08	0.20	0.06	0.18	0.05
	Pooled	0.22	0.04	0.24	0.06	0.18	0.04	0.19	0.04
Bidirectional	3					0.28	0.03	0.18	0.06
	4					0.14	0.03	0.24	0.07
	5					0.15	0.02	0.15	0.04
	Pooled					0.18	0.01	0.17	0.03

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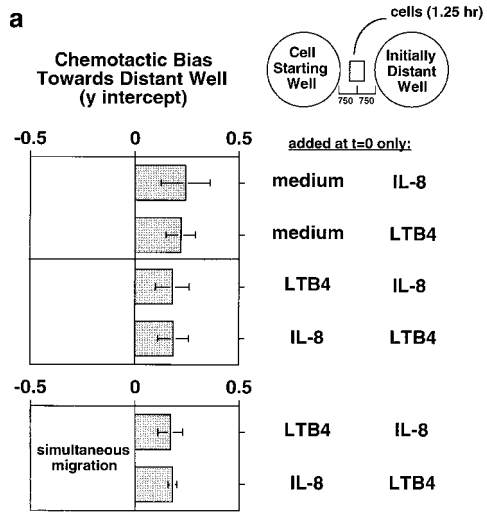

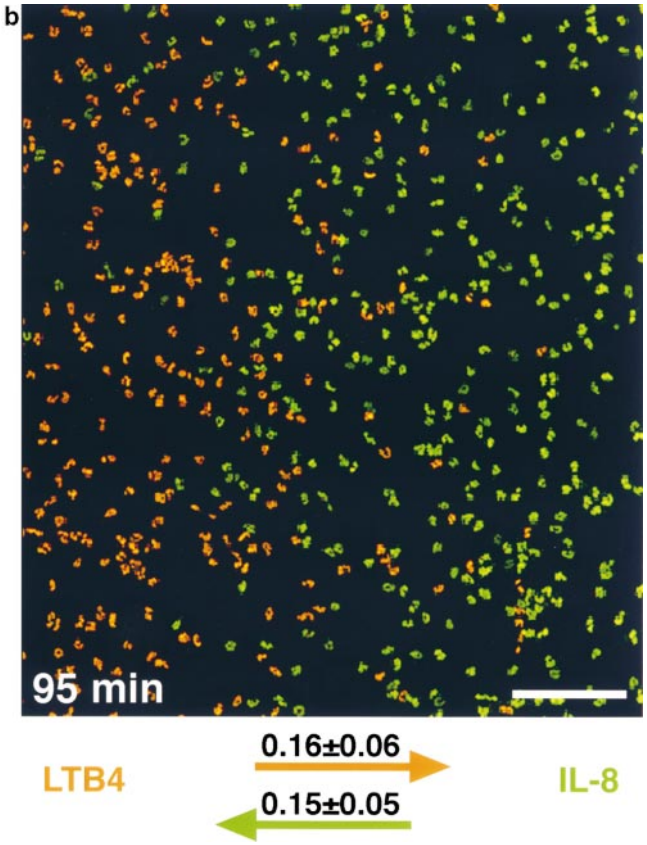

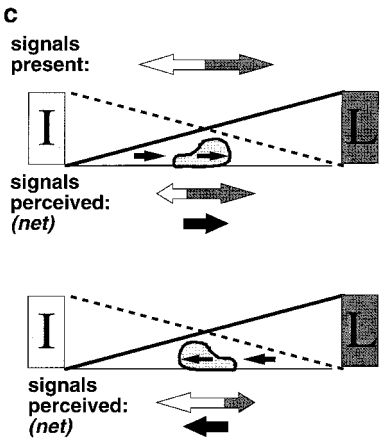

Figure 5. Neutrophils' chemotactic bias depends on the direction from which they have migrated. Neutrophils were tracked as they migrated into a central position between their starting well and a well 1.5 mm away. (a) Chemotactic bias in the central region between two chemoattractant source wells. Chemotactic biases are shown for a single population of migrating cells (top, cells 600–900 μm from each source) and for two fluorescently labeled neutrophil populations allowed to migrate simultaneously (bottom, cells 550–950 μm from each source). (b) Representative micrograph of simultaneous migration at the end of the observation period showing that the two neutrophil populations had intermingled significantly (chemotactic bias of each population in this experiment shown below micrograph). Bars represent the magnitude of cells' chemotactic bias (y-intercept of the initial versus subsequent FMI plot) under different conditions, calculated as a weighted, pooled average from two experiments performed on different days with different blood donors. Error bars show the 95% confidence interval. Results of individual experiments are shown in Table I. Distances from each well to the center of the videotaped field are indicated on the diagram (in micrometers). (c) In this model, cells can vectorially add orienting signals from opposing sources, but cellular perception of orienting signals is determined, in part, by the cell's chemoattractant exposure history. Thus, when cells are migrating in the presence of equivalent chemotactic signals from opposing IL-8 and LTB4 sources, a cell migrating into a central region from the IL-8 direction is less sensitive to the IL-8 signal, and chemotaxes towards LTB4. Conversely, a cell migrating from the LTB4 direction is selectively less sensitive to the LTB4 signal, and chemotaxes towards IL-8. Bar, 100 μm.

Our analysis revealed that neutrophils display true chemotaxis to a distant chemoattractant source, even when the local agonist source also produces an effective (orienting) reverse gradient at the same position and time.

Migrating neutrophils are polarized, displaying a discrete leading edge and trailing uropod, even in the absence of an orienting gradient (Allan and Wilkinson, 1978; Zigmond et al., 1981; Shields and Hatson, 1985). Gradients of leukocyte chemoattractants are thought to elicit chemotaxis by determining the direction of a cell's leading edge and, hence, the direction of cell locomotion, independent of cell velocity, adhesivity, or other factors. If cells can integrate orienting signals, then in the presence of overlapping gradients, the direction of cell chemotaxis would be expected to reflect the vector sum (the average magnitude and direction of) the orienting signals present. If the chemoattractant gradients are not aligned, as when cells are migrating away from one source towards another, the orienting signals would compete. In this situation, whichever gradient produces a stronger orienting signal would be expected to determine the cells' direction of orientation and, thus, determine the direction of cells' chemotactic response. The apparent ability of cells to ignore a local chemoattractant source, thus, may reflect an integrated re-

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sponse to the opposing orienting signals, but one in which the cells' chemotactic bias is determined preferentially by the (presumably more compelling) distant gradient.

In fact, we were able to demonstrate convincingly that neutrophils can integrate conflicting directional signals by showing that, in the presence of two balanced agonist sources 60° apart, neutrophils migrate furthest in a direction between the agonist sources. This observation not only confirms that most neutrophils can respond efficiently to both of the two agonists used in this study (IL-8 and LTB4), but that at the population level, the direction of cells' displacement is determined by the vector sum of the orienting signals provided by each gradient. In the extreme case, this implies that two opposing gradients (of exactly matched orienting strength) should be able to cancel each other out. In fact, under carefully selected conditions, we have been able to create such a balanced competition in which neutrophils between IL-8 and LTB4 sources behaved as if in the presence of a uniform chemoattractant field (Foxman, 1999).

One prediction from these observations is that in the presence of a stable array of regulatory chemoattractants, cells will eventually find a central region in which opposing orienting signals are perceived as equivalent. Within this region, cells would migrate back and forth until they encountered additional signals. Such signals could include a newly introduced chemoattractant source, or other classes of guidance cues such as counterreceptors for leukocyte adhesion molecules (Loike et al., 1995). Even if other influences were irrelevant, the phenomenon of directional persistence (and of cellular memory see below) would ensure that in this setting cells would be broadly distributed throughout a region of regulatory cell activation, as in a tissue site of inflammation.

Cellular Memory: Leukocytes Prioritize Distant or Novel Chemoattractant Sources

In studying neutrophils' chemotactic behavior in the presence of opposing chemoattractant gradients, we discovered that cells presented with identical chemotactic fields exhibit different chemotactic behavior depending upon their history. Neutrophils arriving in a central region between IL-8 and LTB4 sources displayed opposite chemotactic responses, depending on the direction from which they came, even when neutrophils were added simultaneously to both wells and assessed as they migrated towards each other in the same central location.

If cells' directional bias is determined by the integration of opposing orienting signals, how can neutrophils display different chemotactic biases when migrating in exactly the same gradient conditions? We propose that these experiments define a phenomenon of cellular memory of the recent chemoattractant environment, in which cells' responsiveness to agonists in the recent chemoattractant environment is selectively diminished, ensuring that they can preferentially respond to newly arising chemoattractant sources within tissues.

What is the basis of this change in cellular responsiveness? Neutrophils and other leukocytes are known to adjust their sensitivity to a chemoattractant upon exposure to that chemoattractant. At the chemoattractant concentrations that efficiently elicit chemotaxis (0.01–10× the receptor K_d), the conditions most relevant to our experiments, cells undergo a process known as adaptation. Adaptation is a feature of many sensory systems using G protein–coupled receptors, including animal visual systems (Koshland, 1980), and is thought to reflect adjustment in agonist-specific signaling mediated by homologous receptor desensitization (or resensitization and receptor recycling). An adapting system adjusts its sensitivity according to the background level of stimulation it receives. Early studies showed that neutrophils experiencing slight increases in chemoattractant level exhibit a transient response, and then adjust to the new chemoattractant concentration (Zigmond and Sullivan, 1979). This adaptation takes many seconds to several minutes, after which neutrophils can again respond to an additional step increase in chemoattractant concentration. However, the dose of chemoattractant required to elicit a neutrophil response increases as cells adapt to higher ambient chemoattractant concentrations (Seligmann et al., 1982).

Cells that have adjusted to a certain chemoattractant concentration can regain their prior sensitivity to lower agonist levels when the chemoattractant is removed, but this process takes time. Neutrophils that experience a sudden decrease in chemoattractant concentration show morphological changes, which subside within 2–6 min, depending on the magnitude of the decrease (Zigmond and Sullivan, 1979). The full recovery of chemotactic responsiveness can take even longer. For example, in studies by Goldman and Goetzl (1984), neutrophils preincubated with low, chemotactic levels of LTB4 (0.3, 1, or 3 nM), washed, and allowed to recover, showed a significant reduction in chemotactic responsiveness to LTB4 even when assayed starting 10 min later. Cells preincubated with slightly higher concentrations (10–30 nM) exhibited no chemotaxis to LTB4 even when assayed after a 10-min recovery period (Goldman and Goetzl, 1984). Cells that have experienced extreme, saturating chemoattractant levels (~100× the receptor dissociation constant, or K_d) undergo not only receptor desensitization, but also extensive receptor internalization, and can require a long recovery time (20–60 min) to regain former receptor levels (Sullivan and Zigmond, 1980; Zigmond et al., 1982; Samanta et al., 1990; Chuntharapai and Kim, 1995) and signal transduction efficiency (Wilde et al., 1989). We hypothesize that the time delay required to readjust cellular sensitivity after a reduction in ligand exposure is the basis for the phenomena we observe, in essence altering cells' perception of the relative strength of local chemotactic signals as a function of the cells' recent chemoattractant environment. A model is shown in Fig. 5 c.

Temporal Versus Spatial Components of Gradient Perception

How can this model explain the apparent ability of cells to migrate between two chemoattractant sources presented at an angle? Leukocytes and other cell types, such as *Dictyostelium* amebas, are thought to sense chemotactic gradients using a spatial mechanism, in which they instantaneously calculate the differential occupancy of chemoattractant receptors across the cell body (Zigmond,

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1974; Zigmond, 1977; Devreotes and Zigmond, 1988). Although distinct chemoattractant receptors could theoretically each initiate a unique signaling cascade leading to chemotaxis, current studies suggest that signals through different chemoattractant receptors converge on a common chemotaxis-initiating pathway (Arai et al., 1997; Neptune et al., 1999). If different chemoattractant receptors share common intracellular signaling pathways, at any given instant a cell is not likely to be able to tell whether the signals it is receiving are from one agonist or another. Thus, at any instant, neutrophils presented with competing gradients should respond as if in the presence of a single agonist.

However, the cells' ability to adjust their sensitivity to different attractants independently can allow vector integration of distinct orienting signals over time. In our model, when two sources of the same chemoattractant are present, a cell simply migrates up the steepest local gradient it encounters. When two different chemoattractants are present, a cell also migrates up the steepest local gradient. However, as it migrates closer to one chemoattractant source and becomes less sensitive to that agonist and, thus, relatively more responsive to the other, it will turn and move towards the second agonist, potentially even across the midline. Thus, the perception and response to complex chemoattractant arrays involves both instantaneous spatial gradient perception at each moment, and changing perceptions of individual gradient vectors over time. In this model, it is the temporal component that allows cells to integrate orienting vectors from different chemoattractant sources.

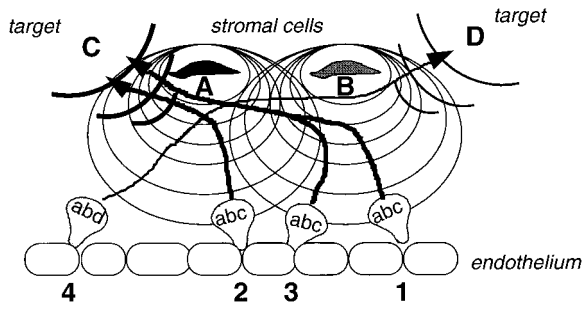

Figure 6. Cellular memory can guide leukocyte navigation through complex chemotactic fields. Leukocytes migrating into a complex chemotactic field navigate to their targets using cellular memory. Cells expressing chemoattractant receptors (a–c) for ligands A–C can navigate to their targets, regardless of where they enter the tissue. A cell that enters near the stromal cell secreting agonist B first migrates up the B gradient (cell 1). As the cell migrates within range of the A gradient, loss in cellular sensitivity to B enhances the cell's migration towards A. Following the A gradient draws the cell close enough to perceive a dominant attractant (C) from its end target. A cell that enters between the two stromal cells may initially migrate up the steepest local gradient it encounters. If it migrates up gradient A (cell 2), it quickly approximates the gradient of the dominant agonist C, which directs it to its target. If it migrates up gradient B (cell 3), any loss in sensitivity to B will increase the influence of gradient A. As the influence of A increases, the cell may migrate within range of the dominant agonist, C, and be attracted towards it destination. Note that if both stromal cells secreted attractant B, cell 3 would likely continue up the steepest local B gradient, and would be unlikely to wander within range of the chemoattractant from its target (C). This system is resilient: if the end target were to move to a site near the stromal cell secreting B, cells could be easily shunted towards the new target. Similarly, stromal cells secreting A and B could recruit cell 4, a different leukocyte subset (expressing receptors a, b, and d), to a target site near stromal cell B. In the absence of an end target, cells would be expected to linger between agonists A and B, as they would become relatively more sensitive to one of the agonists as they approached the source of the other agonist.

The Significance of Cellular Memory for Leukocyte Homing In Vivo

The phenomenon of cellular memory has fundamental implications for directing leukocyte homing in vivo, namely, whenever a cell experiences competing attractant gradients, memory will promote cell migration towards a novel chemoattractant. This model is outlined in Fig. 6.

In this study, we show that neutrophil chemotaxis in overlapping chemoattractant gradients is guided by vector integration of orienting signals, and by cells' memory of their prior chemoattractant environment. (In vivo, of course, these mechanisms would operate in conjunction with regulated adhesion and cell activation to control cell direction.) Our observations support a model in which a leukocyte's chemotactic bias in competing chemotactic gradients is dynamically regulated by its previous experiences. This type of regulation can promote leukocytes' sequential migration to (and navigation through) the gradient sources present within a tissue, allowing combinations of chemoattractants to effectively guide cells to their unique destinations.

We thank Bill Brown for enthusiastic and helpful assistance with statistical analyses and Sally Zigmond, Eddie Bowman, and Dan Campbell for critical review of this manuscript.

This work was supported by grants from the National Institutes of Health (NIH) and an award from the Department of Veterans Affairs (to E.C. Butcher). Ellen F. Foxman was supported by the Medical Scientist Training Program (grant number GM07365) from the National Institute of General Medical Sciences. Eric J. Kunkel was supported by the NIH Immunology Training Grant (grant number 5T32-AI07290).

Submitted: 1 June 1999
Revised: 2 September 1999
Accepted: 27 September 1999

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