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Chemotaxis of Human Polymorphonuclear Leukocytes under Agarose: Lack of Requirement for Media Protein and Differential Effects of Buffer and Agarose Type on Locomotion (41190)

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Abstract. Migration and chemotaxis of human polymorphonuclear leukocytes (PMN) under agarose was studied in the presence and absence of various proteins. Contrary to previous reports, chemotaxis could be elicited in a protein-free medium using both complement-derived factors and fMet–Leu–Phe. Cell movement was critically dependent on the choice of agarose and buffer system. Addition of human serum or albumin produced marked chemokinesis while gelatin had no effect. These results indicate that media protein is a methodological requirement of some *in vitro* test systems but not a physiological requisite for PMN chemotaxis. The described procedures provide a valuable control for studying protein effects on cell migration as well as a means of avoiding unwanted cell stimulation and inactivation of test substances by media protein. This should facilitate studying chemokinetic and chemotactic materials, helper factors, and pharmacological agents. Agarose types were found to differ in the occurrence of a concentration dependent inhibition of locomotion. This suggests that restriction of cell movement by agarose may influence migration of PMN with abnormalities possibly unrelated to locomotion (e.g., cell swelling and decreased deformability induced by pharmacological agents or disease). Conditions are described which should minimize or enhance detection of such phenomena.

Chemotaxis, the ability of cells to migrate directionally in a chemical gradient, has been implicated as a fundamental mechanism whereby polymorphonuclear leukocytes (PMN) accumulate at inflammatory sites (1–4). Our understanding of the phenomenon has developed largely from *in vitro* studies, following the introduction by Boyden in 1962 of a procedure for quantitating PMN chemotaxis through membrane filters (5).

In the Boyden method and its numerous modified forms, and in the more recently described techniques for studying cell migration under agarose (6–9), whole serum or albumin are routinely incorporated in assay media to facilitate locomotion. The availability of a method which avoids the use of media protein would help to clarify a number of questions. It has been reported that in the absence of protein, chemotaxis does not occur (8, 10), suggesting a possible physiological role for extracellular protein (11). Such observations have led to theories that chemoattractants must be bound to a carrier protein in order to activate cell receptors (12), although more recent studies suggest that proteins enhance locomotion of cells by modulating their interaction with artificial substrates (13) and therefore indirectly facilitate chemotaxis. Studies of chemoattractants, helper factors (14), and pharmacological agents (15) are complicated by possible interaction with media protein in addition to the variable and uncertain effects on PMN locomotion of proteins themselves.

We report herein that random migration and chemotaxis of PMN can be elicited in a protein-free environment using modifications of the agarose method, and have compared these results to responses in the presence of three commonly used sources of protein. Two chemotactic factors were studied; Zymosan-activated serum (ZAS), as a source of complement-derived chemotactic activity (16), and the oligopeptide, *N*-formyl methionyl–leucyl–phenylalanine (fMet–Leu–Phe) which acts on a different receptor, in common with bacterially de-

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rived chemotactic factors (17, 18). In addition, physical differences between agarose types were observed which have implications for the study of PMN abnormalities using the agarose method.

Materials and Methods. All materials were obtained and procedures carried out according to Nelson *et al.* (19) except as indicated below.

Source of materials. Ficoll–sodium diatrizoate (Ficoll–Paque, Pharmacia Fine Chemicals, Piscataway, N.J.), HSA, and Hepes (human serum albumin—Fraction V, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, respectively, Sigma Chemical Co., St. Louis, Mo.), gelatin (Difco Laboratories, Detroit, Mich.), Seakem ME agarose (Marine Colloids, Rockland, Maine), Litex HSA agarose (Accurate Chemical and Scientific, Hicksville, N.Y.) fMet–Leu–Phe (synthesized at Abbott Laboratories, North Chicago, Ill.), and PHS (pooled human serum, male AB+, Interstate Blood Bank, Memphis, Tenn.) were used.

Media preparation. The tissue culture media [Earle's balanced salt solution and Spinner's modified MEM (S-MEM) both without bicarbonate] were buffered with 20 mM Hepes and adjusted to pH 7.0 at 37° except where indicated under Results. When Hepes was replaced by bicarbonate buffer (9 meq/liter) the ambient CO_2 concentration was varied according to the barometric pressure to obtain pH 7.0. All solutions were prepared in advance, adjusted to give a final osmolality of 300 mOsm/kg and stored frozen.

On the day of an experiment, agarose was dissolved in water at 2× final concentration using a boiling water bath and cooled to 50° before use. Gelatin was similarly prepared as a 20× solution and diluted 1 part in 9 parts of 2.22× agarose. Buffer solutions at 2× concentration (with or without added protein) were equilibrated at 37° and agarose was added in a 1:1 ratio immediately before dispensing into culture dishes (5 ml/dish). In the experiments using Litex agarose, all solutions were mixed at 50° because of its higher gelling temperature. Final agarose concentrations were 1.0% for Seakem ME and 1.2% for Litex HSA, except where indicated. The plates were kept at room temperature in plastic wrap prior to cutting wells (3 mm diameter, 3 mm apart).

Cell preparation. Thirty milliliters of blood was drawn from four healthy human volunteers and mixed with preservative-free heparin (10 units/ml). Cell purification was carried out essentially as described by Nelson *et al.* (19) using gravity sedimentation of buffy coat cells in autologous platelet-poor plasma (30 min at 37°) followed by centrifugation, resuspension of the leukocytes in S-MEM, and separation of PMN in a Ficoll–sodium diatrizoate density medium (40 min at 400*g*). The purified PMN were washed three times in S-MEM and suspended in media identical in composition and protein content to those used in the agarose plates.

Chemotactic factor preparation. Zymosan-activated pooled human serum (ZAS) was prepared as previously described (20). For use in one experiment, ZAS was enriched for chemotactic activity by HCl precipitation as described by Gerard and Hugli (21) and designated as ZAS–HCl. Pooled human serum (PHS) heat-inactivated at 56° for 30 min served as a control. Aliquots of the above were stored frozen until use. fMet–Leu–Phe was freshly prepared for each experiment by dissolving it in the buffered culture media.

Assay procedure. The random migration and chemotaxis of each donor's cells were measured in duplicate by adding 10 μl of PHS or ZAS, respectively, to the outer wells. Pooled human serum was added to the inner wells. For fMet–Leu–Phe, 10 μl of the peptide solution was used in place of ZAS and the remaining outer and inner wells were filled with buffer. Cells (2.5×10^5/well) were added to the middle wells and the plates were transferred to a humidified incubator at 37° for 2 hr. The plates were then fixed by addition of 2.5 ml of a mixture of 1 part formaldehyde with 9 parts methanol and allowed to stand overnight. The agarose was removed and the cells stained with Wright's Stain.

Cell migration distances were measured using a calibrated eyepiece micrometer in an inverted microscope at 50× magnifica-

tion. For both random migration and chemotaxis assays, the distance from the edge of the cell well to the leading front of migrating cells was measured on the axis between the center of the cell well and the outer well of each assay. The 10 cells farthest from the cell well were excluded to minimize the effect of frontrunners on the magnitude of the measurement. Chemotaxis was assessed by subtracting the distance obtained from the separate random migration control from the chemotactic response to determine the chemotactic difference or by calculating the ratio of these two distances. Group means and standard errors were calculated for each parameter and differences between groups were tested for statistical significance ($P < 0.05$) using Duncan's Multiple Range Test (22).

Results. *Effect of agarose type and concentration.* Both Seakem ME and Litex HSA agaroses were tested at doubling concentrations from 0.5 to 2.0% in Hepes-buffered media containing 10% PHS (Fig. 1). This range essentially spans the working limits of both materials; at lower values the gel is too soft for cutting uniform wells, and at higher concentrations, gelling occurs before plates can be poured. The results reveal a basic difference between the two agaroses. Using Litex HSA, both random migration and chemotactic migration distances were significantly inhibited by increasing the concentration. With Seakem ME, there was a visually apparent reduction in the number of migrating cells at the high concentration, but only a slight decrement in the random migration distance. (The random migration distance using 2% Seakem was significantly lower than at 1%, but did not differ from that at 0.5%.) The net chemotactic difference was unaffected by changes in concentration. A similar response pattern was obtained when these agaroses were tested using a CO_2–bicarbonate buffer system rather than Hepes except that overall migration distances were slightly greater (data not shown).

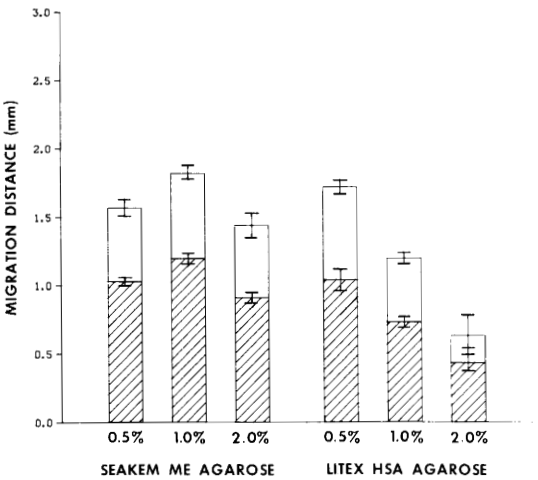

FIG. 1. Effect of agarose type and concentration on PMN response to ZAS. Random migration (▨); chemotaxis (□); medium = Hepes buffer with 10% PHS; bars represent mean ± SEM, $N = 8$.

Effect of media composition on cell response. Random migration and the chemotactic response to ZAS and fMet–Leu–Phe (1×10^{-7} M) were evaluated simultaneously using media in which the presence and source of protein, agarose type, and buffering system were varied (Fig. 2). Protein concentrations which gave a maximal chemotactic difference in pilot experiments using ZAS were selected as being optimal for this comparison study. Using HSA as a source of protein (Fig. 2B), the random migration was generally greater in distance and in cell number than with PHS (Fig. 2A), while the chemotactic response to both chemoattractants was less in the presence of HSA as compared to PHS.

Although there was a tendency for the distance of random migration and chemotaxis to be somewhat greater when using a bicarbonate buffer as opposed to Hepes, despite equivalent pH conditions, this was usually not statistically significant. Agarose concentrations of 1% for Seakem (10, 23) and 1.2% for Litex (19) were selected as representing standard conditions reported by other investigators. Responses were uniformly greater and, in general, significantly so, with cells under 1% Seakem as opposed to 1.2% Litex agarose. This was probably the result of a physical difference between the two agarose preparations. Compared to 1% Seakem, cell responses were inhibited using Litex agarose at 1% or greater concentrations in a previous study (Fig. 1).

With no protein in the media, random mi-

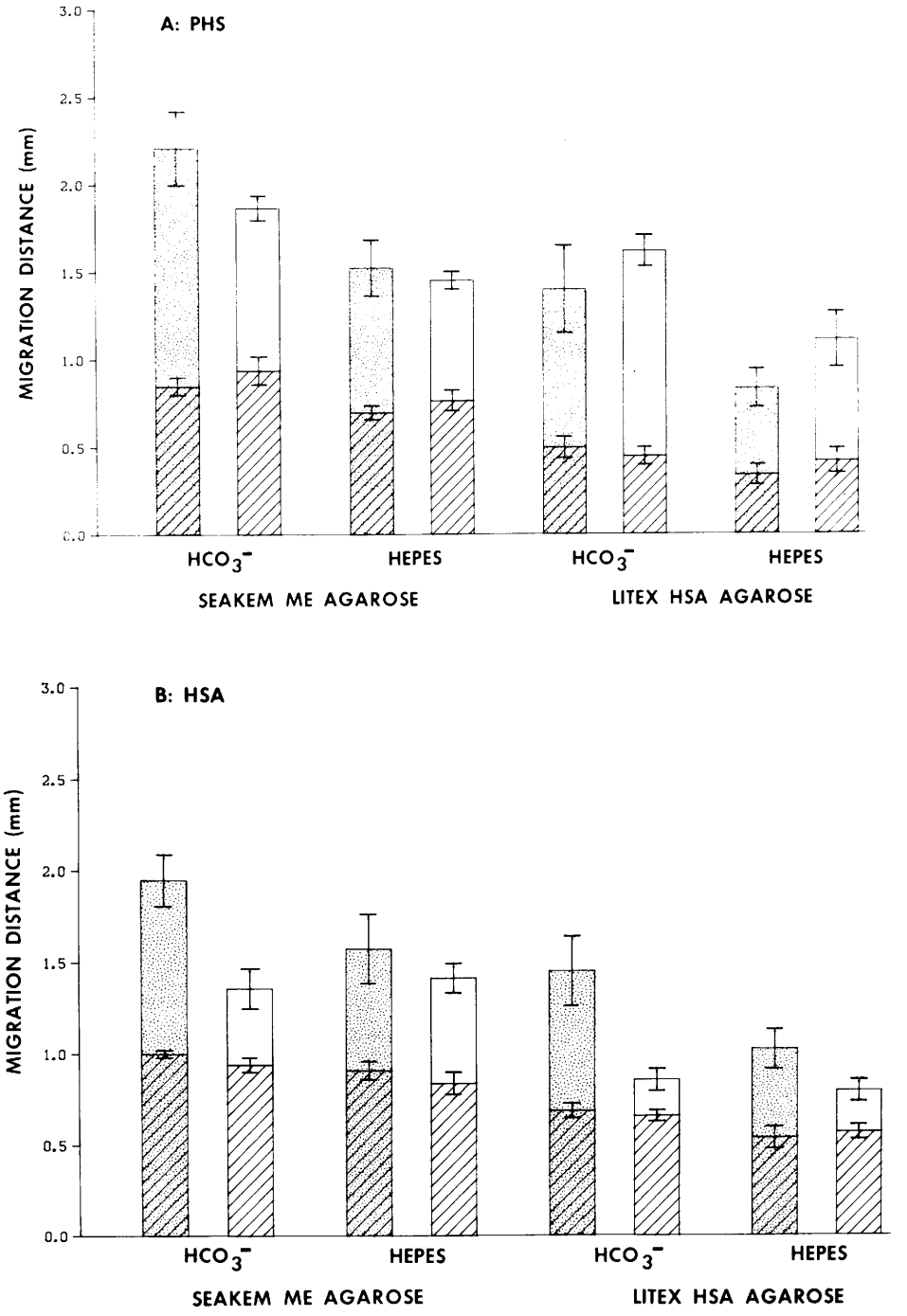

FIG. 2. Effect of protein source, buffer system, and agarose type on PMN response. Random migration (▨); chemotaxis (□); chemoattractant: Shaded bars, fMet–Leu–Phe, 1×10^{-7} *M*; unshaded bars, ZAS; Seakem ME = 1%, Litex HSA = 1.2%; bars represent mean ± SEM, $N = 8$.

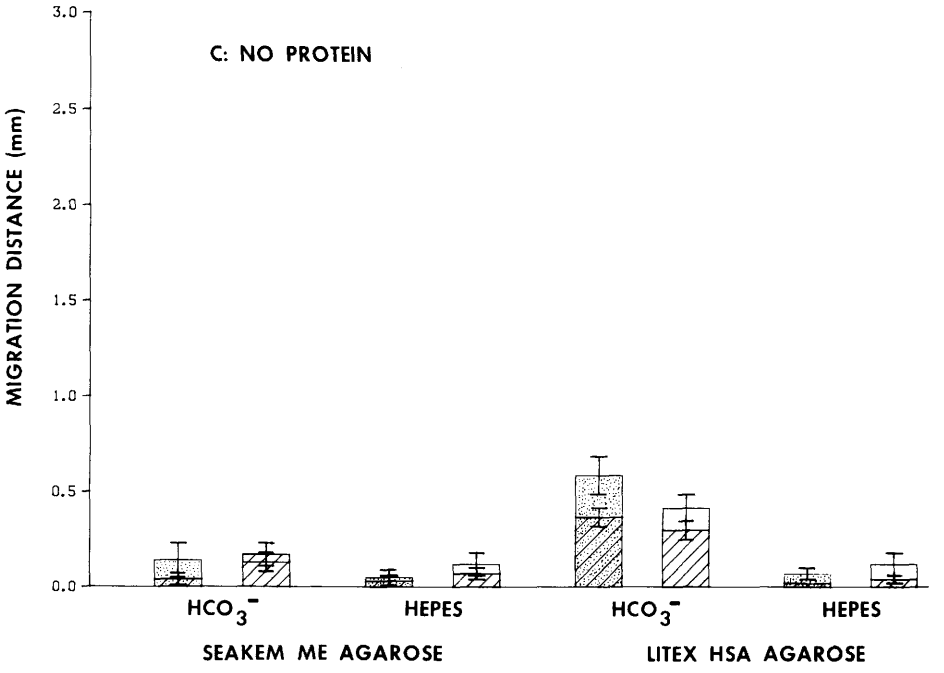

FIG. 2.—*Continued*

gration and chemotaxis were negligible (Fig. 2C). As reported by Nelson *et al.* (8), the agarose layer was difficult to remove after fixation, adhering tenaciously to the petri dish, which suggests that cell movement was physically restricted. However, when Litex agarose was used with bicarbonate, this effect was not seen, and modest but statistically significant random migration and chemotaxis took place. Along with distance, cell densities were less than with either protein source. No other tested agarose–buffer combination allowed significant cell movement without protein.

The use of gelatin compared to no-protein was evaluated in a separate study (Fig. 3). The concentration of 0.25% recommended by Chenoweth *et al.* (23), was found in pilot studies to give a maximal chemotactic difference. With gelatin, the cell response was little different from that of the best no-protein group except that it occurred with all agarose–buffer combinations. In these groups, the gel layer was freely detachable from the plates after fixation, suggesting that the gelatin prevented firm adherence of the agarose.

Chemotactic response using media with no protein. Polymorphonuclear leukocytes were tested (using 1.2% Litex HSA agarose and bicarbonate buffer) without the presence of protein to determine their ability to respond to a variety of chemoattractants (Fig. 4). Because random migration distance and cell numbers were much lower under this condition, the chemotactic ratio was used to compare results with and without protein.

As in the previous study without protein (Fig. 2C), ZAS produced a modest but statistically significant chemotactic response. Three- to five-fold increases in the ratio were obtained using more concentrated chemoattractant (ZAS–HCl) prepared from ZAS according to the method of Gerard and Hugli (21). This material was previously found to have approximately seven times the chemoattractant activity of ZAS when compared using media containing 10% PHS (data not shown). fMet–

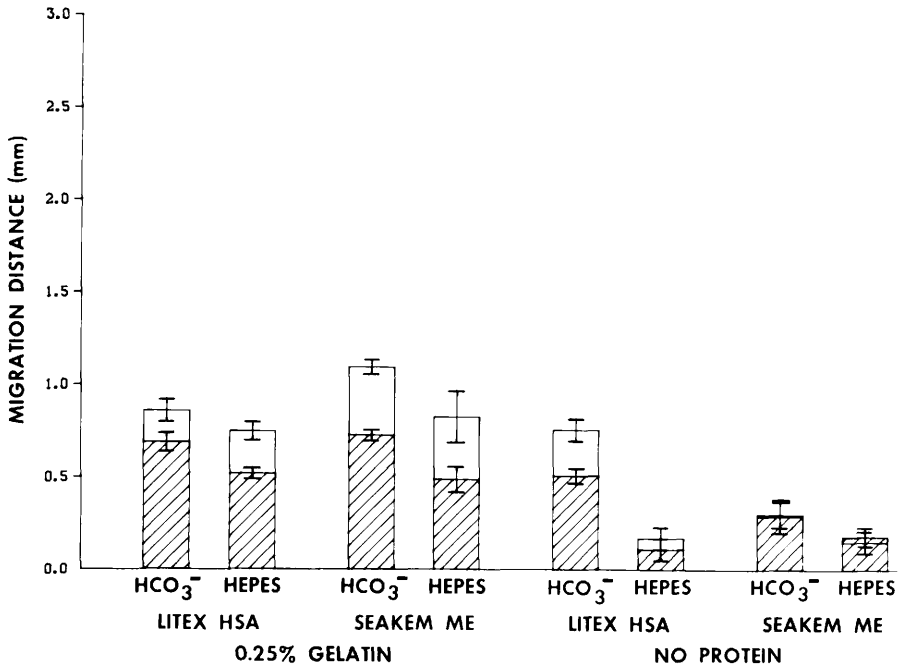

FIG. 3. Comparison of gelatin versus no protein on PMN response to ZAS. Random migration (▨); chemotaxis (□); Seakem ME = 1%, Litex HSA = 1.2%; bars represent mean ± SEM, N = 8.

Leu–Phe produced no effect at 1×10^{-8} M, but gave results equal to and twice the control ratio at 1×10^{-7} and 1×10^{-6} M, respectively.

Discussion. Protein has been reported to be an essential requirement for studying PMN locomotion under agarose (8, 10). Those findings may be attributable to the type of agarose used or an insufficient concentration of attractant. Our results have clearly demonstrated that under suitable conditions, PMN are capable of random migration and chemotactic responses to both ZAS and fMet–Leu–Phe in the absence of media protein (Figs. 2C and 4). [Throughout this report we have described the response to these agents as representing chemotaxis because the cells were exposed to a concentration gradient of attractant. In the agarose method, as in filter techniques, stimulation of random movement (chemokinesis) by an attractant can contribute to the apparent chemotactic migration distance (24)]. It has been shown by Repine and Clawson (11) that cell-adherent plasma proteins can compensate for a lack of media protein unless removed by multiple washings. This possibility should have been avoided in the present study by the use of a 3× wash procedure. The major differences in response without protein were that fewer cells migrated, resulting in lower random and chemotactic migration distances (Figs. 2C and 3), and that eliciting appreciable chemotaxis required higher concentrations of attractant than with PHS (Fig. 4). These phenomena may be related due to the diffusion characteristics of the assay system. The agarose method has been reported to be 100 times less sensitive than filter methods in detecting chemoattractants (25) which may be a result of the much greater diffusion distance between cells and attractant (3 mm versus 0.15 mm). From the start of incubation, random migration brings the cells closer to the attractant well and may be an important factor in determining whether cells receive a sufficient chemotactic stimulus. Because of the low random migration in the present studies without protein, higher chemoattractant concentrations may have been required in

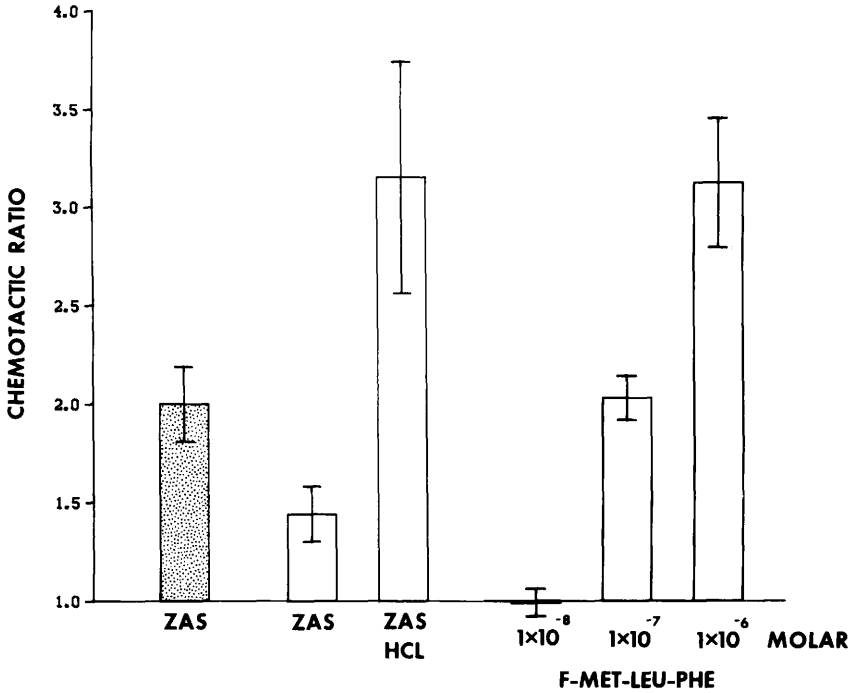

FIG. 4. Chemotaxis of PMN with no protein. Chemotactic ratio = (chemotactic migration distance)/(random migration distance). Shaded bar: control using 10% PHS in media; open bars: no media protein; all groups = bicarbonate buffer and 1.2% Litex HSA agarose; bars represent mean ± SEM, $N = 8$.

order for an effective stimulating concentration to reach the leading front of migrating cells within the 2-hr incubation period.

The fact that significant cell migration occurred without protein only when using Litex HSA agarose (Figs. 2C and 3) seems attributable to a physical difference from Seakem ME agarose under these conditions. That these materials have basic physical differences is further indicated by our results suggesting that Litex HSA agarose can physically restrict cell movement. This was observed when comparing the two agaroses at typical, although differing, concentrations described in the literature (see Fig. 2 and Results) as well as at equivalent concentrations (Fig. 1). Why cell movement is limited by an increase in agarose concentration is not clear. It may be due to an increase in the stiffness of the gel or its adherence to the substrate (which exceeds the ability of the PMN to deform it as they migrate) or a reduction in the space between the gel and the surface of the dish (which exceeds the limits of cell deformability). In a recent study, nonsegmented PMN from patients with bacterial infections exhibited marked inhibition of locomotion in a Boyden assay but had normal migration and chemotaxis when tested under agarose (26). The inhibition was attributed to pore size restriction of cell movement by the membrane filter. This physical effect of restricting migration of cells with less than normal deformability has also been reported to explain the poor *in vitro* responses of PMN from patients with Chediak-Higashi syndrome (27) and Pelger-Huet anomaly (28), which were previously thought to result solely from defective cell locomotion. Our observations with the agarose assay indicate that the choice of agarose and its concentration can potentially influence the interpretation of such studies. The marked difference between Litex HSA and Seakem ME agarose suggests that in order

to minimize the possible influence of abnormal cell deformability on PMN locomotion, the agarose employed should be determined to be relatively free from concentration-dependent restriction of cell movement. Seakem ME agarose appears to be superior to Litex HSA in this regard. If high concentrations of Litex HSA do inhibit PMN locomotion through physical restriction, its use in the agarose assay may provide a unique approach to the detection and study of abnormal deformability and possibly other PMN defects.

Comparison among proteins indicated that using PHS as a protein source generally gave a superior chemotactic difference (Fig. 2A). Random migration was greater with HSA but chemotaxis was less (Fig. 2B). Opposite results for these two materials were reported by Repo (10), who stated that the use of HSA is preferable. Both materials greatly enhanced random migration as compared to no protein. These differences may reflect the inherent difficulty in standardizing techniques based on heterogeneous materials. We have found that random migration and chemotactic differences attainable with ZAS can vary considerably among different lots of PHS (data not shown). The process of heat inactivation routinely employed in preparing PHS may itself generate substances which stimulate PMN locomotion (29). It is also known that fresh human serum contains chemotactic and chemokinetic factors (30, 31), and further, that exposure of untreated serum to agarose can activate the complement system (8). Although the latter properties of serum are reportedly heat sensitive, there may be sufficient variation in lability among some donor sera to leave detectable residual activity. Studies with albumin indicate that it can have direct effects on the cell membrane which modulate locomotion (32). Several investigators have reported that albumin, when used alone, has chemokinetic properties, but without directly comparing it to other sources of protein (13, 33). The characteristic response we obtained with HSA as compared to PHS, greater random migration with a lower chemotactic difference, supports this hypothesis.

It is clear that while these proteins may have utility, their use can add significantly to the complexities of basic studies of PMN locomotion. The need for a pure and chemically well-defined protein source for this purpose may have been better met by Chenoweth *et al.* (23) who have reported good results with gelatin. We have found, however, that using gelatin with the present assay system gives results which are little different than without protein (Fig. 3). The poor performance of gelatin in the present study may be due to the use of a plastic surface rather than the gelatin-pre-coated glass recommended by Chenoweth *et al.* (23). In addition, our use of a larger well size and interwell distance (3.0 mm in the present study versus 2.5 mm) would be expected to lower random migration by decreasing the cell density in the well and provide less chemotactic stimulation.

Our results suggest that in the agarose system, media protein serves two purposes. First, preventing firm adherence of the agarose to the substrate which allows cell movement. Second, to the extent that the protein has chemokinetic properties, a greater chemotactic difference may result whether the chemokinetic effects are due to enhanced cell–substrate interaction or some other mechanism. This is because the greater random movement brings the leading front of cells closer to the source of chemoattractant, allowing them to respond earlier and thus migrate directionally over a greater distance within a given time.

The above findings indicate that best results for studying PMN migration and chemotaxis under agarose in the absence of protein would be achieved through use of the following: (1) Litex HSA agarose with a bicarbonate buffer; (2) the highest feasible cell concentration in order to increase random migration; and, (3) a minimum interwell distance, a longer incubation time and preincubation of plates with the chemotactic factor to increase cell exposure to chemoattractant during the assay.

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