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Neutrophil Responsiveness to Chemoattractant Tripeptide in Rheumatoid Arthritis (42591)

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Abstract. Neutrophils isolated from medication-free rheumatoid arthritis (RA) patients were assayed for responsiveness to the bacterial chemoattractant tripeptide formyl–methionyl–leucyl–phenylalanine (f–Met–Leu–Phe). Rheumatoid arthritis neutrophil preparations contained significantly lower percentages of rapidly migrating cells. This relative hyporesponsiveness of RA neutrophils was related to impaired sensing of chemotactic gradients. Rheumatoid neutrophil abnormalities in sensing of and responding to chemotactic gradients were not associated with resting or f–Met–Leu–Phe-induced changes in arachidonic acid metabolism.

Rheumatoid arthritis (RA) may predispose patients to increased susceptibility to systemic (1–3) and localized (4–7) bacterial infections. Increased susceptibility to infections in patients with diabetes, burns, or the persistent lymph node syndrome has been associated with defective chemotactic responsiveness to the bacterial tripeptide formyl–methionyl–leucyl–phenylalanine (f–Met–Leu–Phe; f-MLP) (8–10). Since previous studies of neutrophil chemotaxis in RA have not been well controlled for medication effects (11–15), have not utilized methods for tracking all responding cells (16–18), and have not utilized quantifiable doses of f–Met–Leu–Phe, we have modified existing methods to determine the functional distribution of all neutrophils in untreated RA patients responding to this standard chemoattractant.

Materials and Methods. *Clinical study protocol.* Patients with active definite or classical RA who were stable under treatment with nonsteroidal agents but who had not received additional antirheumatic medication for at least 3 months were enrolled in these studies. All patients underwent a 2-day to 2-week washout of all medications including nonsteroidal anti-inflammatory agents. Results obtained in RA patients were compared with those obtained in normal controls.

Clinical and laboratory evaluation. Blood for neutrophil studies was drawn from all RA patients at the end of the washout period. Clinical and laboratory data were obtained to calculate a modified rheumatoid activity index as previously described (19). Routine blood chemistry, hematologic studies, urinalyses, and stool occult blood determinations were also performed.

Cell isolation. Neutrophils were isolated from venous blood drawn from RA patients and healthy volunteers with modification of methods previously described (20). In brief, the heparinized (10 units/ml) blood was mixed 1:3 with isotonic saline, underlaid with 0.3 vol of histopaque and centrifuged for 45 min at 300*g*. Serum and mononuclear cells were removed and the remaining neutrophil cell layer was mixed with 1 vol of 3% dextran in isotonic saline. After 40 min, the upper layer was drawn off and centrifuged (180*g*, 10 min) yielding a neutrophil-rich pellet. Hypotonic erythrocyte lysis yielded preparations with >95% neutrophils which were >97% viable (trypan blue exclusion test). Cell preparations were washed and suspended at 2×10^6/ml in Hanks' balanced salt solution (HBSS) alone or supplemented with 0.5% bovine serum albumin (BSA).

Chemotaxis assays. Neutrophils were suspended in HBSS–BSA and added to the upper compartments of blind well chemotactic chambers fitted with 3-μm porosity nitrocel-

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lulose filters (Sartorius, Hayward, CA). Lower compartments contained HBSS–BSA with or without 10^{-10} to 10^{-5} M f-Met-Leu-Phe (Sigma, St. Louis, MO). Chambers were incubated at 37°C for 40 min.

Leading front and mean depth analytic methods. Migration of the leading front of cells was automatically tabulated as previously described (21) for RA patients and normal controls. Data for mean depth estimation were determined by counting the number of cells at every 10-μm depth in three 200× fields (17) and further analyzed by calculating the slope of the linear regression plot of the log of the number of cells versus the depth (μm) squared (18). A mean depth ± SEM for triplicate filters was determined for RA patients and for corresponding normal controls from the calculated slopes of migration.

Population ratio determination. Previously reported (16, 18, 22) methods were modified to enhance resolution of migration differences between cell populations. Zigmond has shown that the distribution of a single population of cells moving into a micropore filter is similar to that of a population of randomly moving particles (22). The distribution of cell number N is described by Eq. 1 (16):

$$N = \frac{N_0}{\sqrt{\pi Dt}} e^{(-x^2/4Dt)} \qquad \text{[Eq. 1]}$$

where N is the number of cells at distance x at time t, N_0 is the total number of cells, and D is the diffusion coefficient. In Zigmond's original experiments with equal concentrations of stimulus (horse serum) above and below the filter, plots of log N vs x^2 yielded straight lines, confirming that the toe of the distribution is representative of the whole distribution, and also, of more interest here, that Gaussian-type analysis was applicable to the migration behavior of neutrophils. Under conditions of chemotactic stimulation we have found that plots of log N vs x^2 or $x^{1.5}$ (18) are usually curvilinear as are those obtained for multiple reaction first-order rate constant processes. If we assume that the curvilinear cell migration plots are due to multiple cell populations an estimate of the size of each population can be obtained by deconvolution of the curvilinear data in analogy with multiple rate constant analysis (23). Practically, this means locating the linear portion of the plot and extrapolating it to zero migration (y axis intercept). The values for log N on the extrapolated line are subtracted from the original data to yield a second plot that will be straight if there are only two subpopulations or curved in the case of three or more cell subpopulations. In practice, usually only one deconvolution step is useful. The plot for the second population is also extrapolated to its y intercept. Taking the logarithm of each side of Eq. 1 and casting the result in the form of the equation for a straight line, we find that the y intercept is directly proportional to the total number of cells in a subpopulation, while it is inversely proportional to the square root of the diffusion coefficient D. Thus if D does not vary greatly with x (22), the ratio of these intercepts is a reasonable estimate of the sizes of cell subpopulations. This method does not prove the existence of subpopulations nor does it purport to identify all that may be present. It does, however, allow differences between cell populations to be more easily quantitated and it provides a model that is consistent with observations of cell migration. A representative cell migration profile with regression plots for calculation of fast and slow populations is shown in Fig. 1.

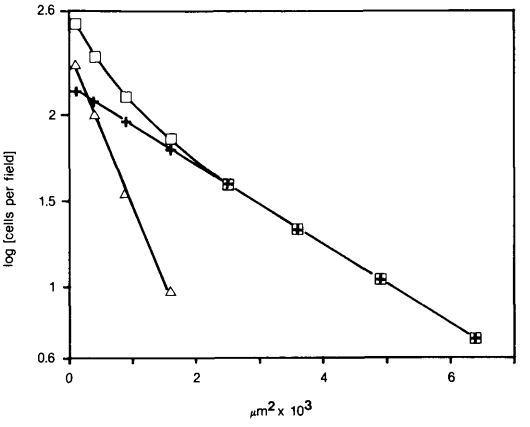

FIG. 1. Chemotactic response of neutrophils from a representative normal healthy volunteer plotted as log (cells/field) vs square of migration distance. Over all migration data (□) toward 2×10^{-8} f-MLP and linear regression plots for fast (+) and slow (△) subpopulations are as indicated with percentage fast cells = 39.0% according to the formula: % fast cells = intercept (+)/(intercept (+) + intercept (△)) × 100.

Comparison of analytic techniques with differing neutrophil populations. Mixtures of untreated and deactivated (10^{-5} *M* f-MLP for 20 min) (24) normal cells were used as models for testing the efficiency of our analytic systems in detecting differences in bimodal neutrophil populations. As shown in Fig. 2, each analytic technique detected significant ($P < 0.01$) differences between untreated cells, deactivated cells, and a 1:1 mixture of each type.

Cell orientation assay. One hundred microliters of neutrophil suspension (2×10^6 cells/ml HBSS–BSA) was transferred to a 22 × 40-mm No. 1 $\frac{1}{2}$ glass coverslip and placed in a humidified incubator at 37°C for 1 hr. The coverslip was washed with isotonic saline, leaving adherent neutrophils. The prepared coverslip was secured, cell-side down, on a Zigmond orientation chamber (25) so that the adherent cells were centered on the bridge of the chamber. One well of the chamber was filled with HBSS–1% BSA, the other with 2×10^{-8} *M* f-MLP in HBSS–1% BSA. After 30–90 min incubation at 37°C in 100% relative humidity, chambers were removed and examined under phase contrast at 400×. One hundred cells on each chamber were classified as nonpolarized or polarized. The numbers of polarized cells directed toward the well containing the chemoattractant were calculated for RA patients and normal controls (25).

Radioimmunoassays of arachidonic acid metabolites. Aliquots (1 ml) of neutrophils suspended in HBSS were added to 1.5-ml microcentrifuge tubes. Aliquots for assay of prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), 5-hydroxyeicosatetraenoic acid (5-HETE), leukotriene B_4 (LTB_4), and leukotriene C_4 (LTC_4) were incubated at 37°C for 10 min either untreated or stimulated with f-Met-Leu-Phe (10^{-7} *M*). All samples were centrifuged 30 sec at 13,000*g*. Supernatants were stored at −70°C under argon. Radioimmunoassays of PGE_2, TXB_2, 5-HETE (Seragen, Boston, MA), and LTC_4 (New England Nuclear, Boston, MA) were performed with kits obtained from the suppliers indicated (26–28). Disintegrations per minute were counted for each supernatant in scintillation fluid (Bray's, New England Nuclear, Boston, MA). Calculations of picograms/0.1 ml were performed with a computer program plotting logit % bound [ordinate] vs log picograms [abscissa] using linear regression analysis. Detection ranges were 8.2–2000 pg/0.1 ml for PGE_2, 8.2–2000 pg/0.1 ml for TXB_2, 12.5–2000 pg/0.1 ml for 5-HETE, and 12.5–1600 pg/0.1 ml for LTC_4. Assays for LTB_4 were performed by

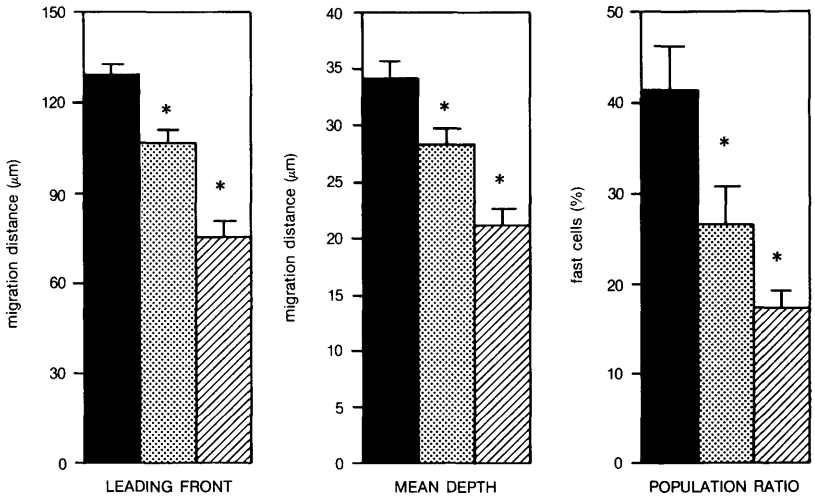

FIG. 2. Comparison of analytic techniques with differing neutrophil populations. All assays performed with blind well chambers 2×10^{-8} *M* f-met-leu-phe in lower compartments, 3 μm porosity, 150-μm thick nitrocellulose filters, 40 min incubation at 37°C. Data shown for normal neutrophils; untreated (solid), preincubated for 20 min with 10^{-5} *M* f-met-leu-phe (hatched), or 1:1 mixture of these (stippled). All data = $\bar{X} \pm$ SEM, $n = 13$; *$P < 0.01$ by paired *t* test.

Dr. Edmond Ku at CIBA–GEIGY Corporation as follows: Specimens were diluted with buffer (50 m*M* Na-phosphate, pH 8.5) to a LTB_4 level of 1–2 ng/ml. Radioimmunoassay was performed with 100-μl aliquots of the diluted samples using radioimmunoassay kits supplied by Amersham Corporation (Chicago, IL) and procedures similar to those reported by Salmon *et al.* (29). All arachidonic acid metabolites were reported as nanograms/10^6 neutrophils.

Statistical analysis. Comparisons among data for normal cells after differing treatments (Fig. 2) were accomplished with a paired Student *t* test. Comparisons among data for RA patients and normal controls were analyzed with an unpaired Student *t* test for samples of equal size.

Results. *Chemotaxis dose-response curves.* Normal and RA neutrophils exhibited maximum chemotaxis in the leading front and mean depth assays when exposed to 10^{-8}–10^{-6} *M* (Figs. 3a and b). Normal neutrophils appeared to mobilize increased numbers of relatively fast moving cells as f-Met-Leu-Phe concentration increased from 10^{-8} to 10^{-6} *M* (Figs. 3a and b). Rheumatoid arthritis neutrophils are somewhat hyporesponsive to optimal concentrations of chemoattractant but significant differences were not detected between RA and normal neutrophils by the leading front or mean depth analytic methods. In contrast, the population ratio analysis revealed differences between RA and normal neutrophils exposed to 10^{-8} and 10^{-6} *M* f-Met-Leu-Phe. The loss of difference between RA and normal neutrophils at 10^{-5} *M* f-Met-Leu-Phe in the population ratio determination (Fig. 3c) is probably a consequence of the small migration distances (Figs. 3a and b) occurring at this chemoattractant concentration because of neutrophil chemotactic deactivation.

Chemotaxis and orientation studies. Neutrophils from larger (n = 21) populations of normal subjects and RA patients were studied with 2×10^{-8} *M* f-Met-Leu-Phe. As seen in Fig. 4, there was no significant chemotactic difference between normal neutrophils and those of the RA patients as measured by leading front (Fig. 4a) or mean depth (Fig. 4b) analytic methods. There was significantly ($P < .05$) less response by the RA cells when the data for the migrating cells were analyzed by the population ratio method (Fig. 4c) with results expressed as the percentage of fast cells among the responding neutrophils.

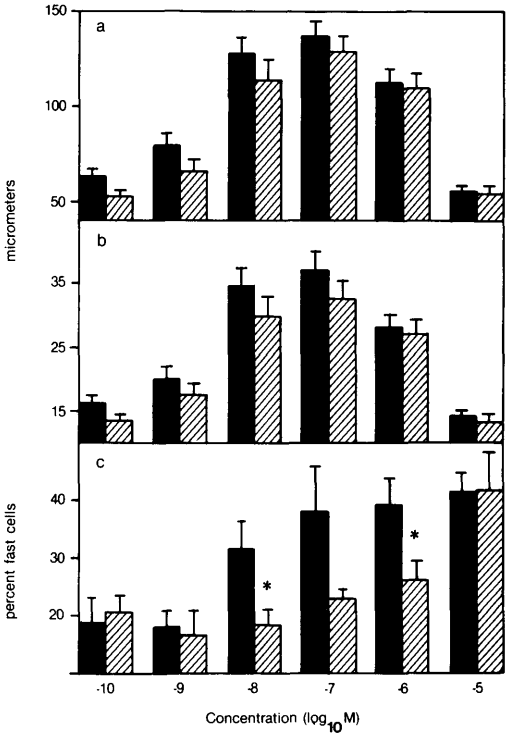

FIG. 3. Dose response of normal (solid) and rheumatoid arthritis (hatched) neutrophils to f-met-leu-phe. Analysis of cell migration by (a) leading front measurement, (b) mean depth estimation, (c) population ratio determination. All data = $\bar{X} \pm$ SEM, n = 6; *$P < 0.05$ by *t* test.

Separate experiments revealed a significant ($P < 0.05$) defect in the ability of RA neutrophils to orient in the direction of a chemoattractant gradient (Fig. 4d, RA $\bar{X} \pm$ SEM = 27 $\pm$ 3; normals $\bar{X} \pm$ SEM = 35 $\pm$ 2). Although polarization (regardless of direction) of RA neutrophils ($\bar{X} \pm$ SEM = 53 $\pm$ 5.9) was less than that of normals (67 $\pm$ 4.0) the difference between these values did not reach statistical significance ($P < 0.05$). The data for percentage fast cells and percentage positive orientation were compared by calculating the ratio of normal to RA values (Figs. 4c and d). This calculation showed that 2×10^{-8} *M* f-Met-Leu-Phe induced normal cells to increase percentage fast cells and percentage positively oriented cells by similar percentages, i.e., 31 and 26%, respectively. As shown in Table I,

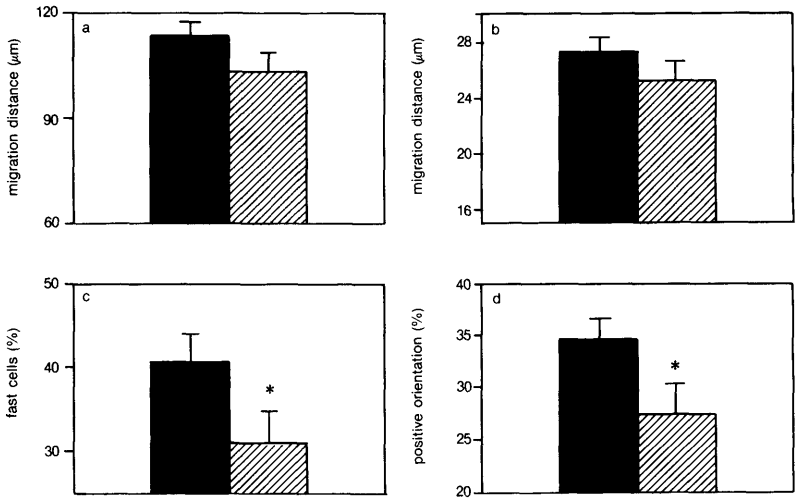

FIG. 4. Comparison of normal (solid) and rheumatoid arthritis (hatched) neutrophil response to 2×10^{-8} *M* f-met-leu-phe. Analysis of cell migration by (a) leading front measurement ($n = 21$), (b) mean depth estimation ($n = 21$), (c) population ratio determination ($n = 21$), (d) analysis of gradient sensing by cell orientation assay ($n = 16$). All data = $\bar{X} \pm$ SEM; $^*P < 0.05$ by *t* test.

leading front and mean depth data correlated extremely well within both the normal control group and the RA group. A less striking but significant ($P < 0.05$) correlation was observed between the population ratio data and the orientation assay values in both the normal control and the RA study groups. Within the RA group there was no correlation of population ratio or orientation data with the type of preexisting nonsteroidal anti-inflammatory agent therapy or the washout period prior to neutrophil isolation for these studies.

Release of arachidonic acid metabolites. Figure 5 shows normal and RA neutrophil production of the cyclooxygenase products prostaglandin E_2 (Fig. 5a) and thromboxane B_2 (Fig. 5b) and the lipoxygenase products 5-HETE (Fig. 5c) and leukotriene C_4 (Fig. 5d). Normal and RA neutrophils did not differ in their production of these eicosanoids and did not increase their production when incubated with 10^{-7} *M* f-Met-Leu-Phe. Similar findings were demonstrated for leukotriene B_4 production by unstimulated neutrophils: normals

TABLE I. CORRELATION OF ANALYTIC MODALITIES WITHIN NORMAL AND RHEUMATOID ARTHRITIS NEUTROPHIL STUDY GROUPS

	Leading front	Mean depth	Population ratio	Orientation assay
	(rheumatoid arthritis patients)			(normal controls)
Leading front ($n = 21$)	—	0.957[a]	0.187	0.404
Mean depth ($n = 21$)	0.973[a]	—	0.132	0.435
Population ratio ($n = 21$)	0.227	0.194	—	0.509[b]
Orientation assay ($n = 16$)	0.370	0.287	0.534[b]	—

[a] $P < 0.001$.
[b] $P < 0.05$.

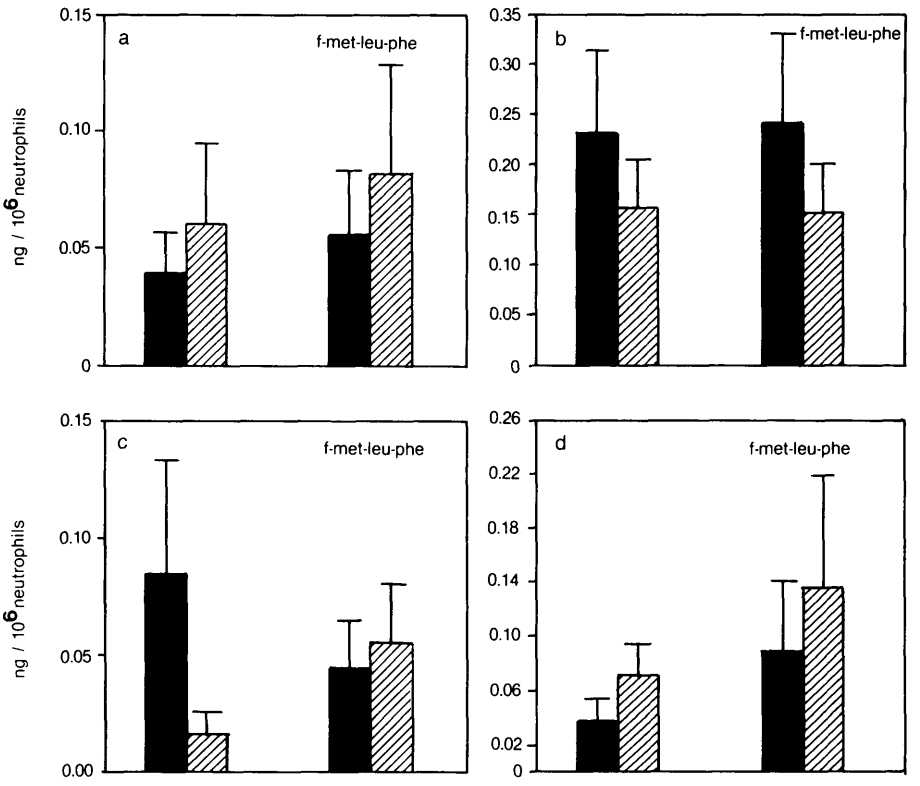

FIG. 5. Effects of f-met-leu-phe (10^{-7} *M*) on eicosanoid production by normal control (solid) and rheumatoid arthritis (hatched) neutrophils. Data shown obtained by radioimmunoassay for (a) prostaglandin E_2, (b) thromboxane B_2, (c) 5-hydroxyeicosatetraenoic acid, (d) leukotriene C_4. All data = $\bar{X} \pm$ SEM, n = 10.

= 0.03 ± 0.02 ng/10^6 neutrophils, and RA patients = 0.02 ± 0.01 ng/10^6 neutrophils. No significant increase in leukotriene B_4 production was observed in the presence of 10^{-7} *M* f-Met-Leu-Phe.

Discussion. The quantitative dose-response studies shown in Fig. 3 (a and b) indicate that RA neutrophils respond normally to 10^{-8} to 10^{-6} *M* f-Met-Leu-Phe in conventional chemotaxis assays. Similar dose-response relationships for f-Met-Leu-Phe and normal neutrophils have been reported in binding, orientation, and chemotaxis assays (30–32). The new population ratio analytic method differentiates between relatively fast and slow cell populations and detects significant differences between normal and rheumatoid populations at 10^{-8} and 10^{-6} *M* f-Met-Leu-Phe (Fig. 3c). These differences may represent greater shifting of cells into the fast population group as normal neutrophils respond to chemoattractant concentrations of f-Met-Leu-Phe. Subpopulations of neutrophils have been described which have differing responses to f-Met-Leu-Phe (33) and other chemoattractants (34).

As expected from the dose-response data, no differences between RA and normal neutrophils were observed in an expanded study at 2×10^{-8} *M* f-Met-Leu-Phe using the leading front measurement (Fig. 4a) and the mean depth estimation (Fig. 4b). On the other hand, small but significant differences were shown by population ratio analysis (Fig. 4c) and by the cell orientation assay (Fig. 4d). These findings demonstrate that RA neutrophils have decreased ability to sense chemotactic gradients of f-Met-Leu-Phe and are hyporesponsive in developing increased numbers of fast-moving cells in these gradients. A linkage

between these two defects is suggested by the similarity of the normal/RA ratio (Figs. 4c and d) for percentage increases in fast cells (31%) and positive orientation (26%). Calculations shown in Table I confirm the correlation between the leading front and mean depth analytic methods within RA and normal neutrophil populations. They also confirm the linkage between the orientation assay and the population ratio measurements within each of these populations.

Neutrophil production of arachidonic acid metabolites was studied because f-Met-Leu-Phe may stimulate neutrophil motility through activation of phospholipase A_2 or arachidonate lipoxygenase (35, 36). The present data (Fig. 5) confirm that human neutrophils possess cyclooxygenase activity (37, 38). While some baseline production of the lipoxygenase products 5-HETE, LTC_4, and LTB_4 was observed, there was, as previously reported (39), no marked increase in production of lipoxygenase products in the absence of exogenous arachidonic acid. Taken together, these results suggest that at a concentration (1×10^{-7} M) which is chemotactic in our assays, f-Met-Leu-Phe does not stimulate normal or RA neutrophils to produce significantly increased amounts of arachidonic acid metabolic products. These data also suggest that differences in tripeptide effects on arachidonic acid metabolism do not account for the chemotactic differences demonstrated between RA and normal neutrophils. The absence of abnormal levels of any arachidonic acid metabolities in resting or f-Met-Leu-Phe treated RA neutrophils is interesting in view of the previously reported (40) elevated levels of 5-HETE in RA synovial tissues.

Since articular inflammation in RA may be a correlate of neutrophil influx into the synovial fluid (41) and the pannus cartilage junction (42), our finding of peripheral blood neutrophil hyporesponsiveness to f-Met-Leu-Phe and the recently reported similar findings with the serum-derived chemotactic factor C5a (43) may result from an eflux of rapidly responding cells (33, 34) into the inflamed synovial structures rather than from an intrinsic hyporesponsiveness of RA neutrophils to chemoattractants. Previous work by our group (44) and others (15, 45) has suggested localized rather than systemic neutrophil problems as the major contributors to the development of septic arthritis in RA patients. Although previous chemotaxis studies are difficult to interpret because failure to control for known medication effects on neutrophil function (15) defective peripheral blood neutrophil responsiveness has been more consistently found in RA patients with Felty's syndrome than in those with RA alone (46, 47). Moreover, recent studies have found no local defects in RA neutrophil migration (48) or in handling of the important pathogen *Staphylococcus aureus* (49). Our careful evaluation of neutrophil responsiveness to the well-defined bacterial chemoattractant f-Met-Leu-Phe supports these findings and describes a novel method for differentiating chemotactically distinct subpopulations of RA peripheral blood neutrophils.

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