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DIFFERENCES IN SUPEROXIDE PRODUCTION BY NONMIGRATING AND MIGRATING HUMAN MONOCYTE SUBPOPULATIONS

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SUMMARY. Human peripheral blood monocytes comprise two subpopulations. Whereas one subpopulation migrates to a variety of chemoattractants in vitro, the remaining subpopulation fails to respond to chemoattractants. The subpopulations were isolated with a chemotaxis separation chamber and their superoxide generation was studied. Superoxide production was continuously monitored during monocyte exposure to phorbol myristate acetate, N-formyl-methionyl-leucyl-phenylalanine and N-formyl-norleucyl-leucyl-phenylalanine. Superoxide production by nonmigrating monocytes was less than 33% of the migrating subpopulations' production. Thus, the subpopulation of monocytes which fails to migrate to chemoattractants also demonstrates defective superoxide production.

INTRODUCTION

Human peripheral blood monocytes contain subsets of cells which can be distinguished on the basis of size (1-3), expression of Fc receptors (2,4), chemotactic responsiveness (1,5), and polarization in a chemoattractant (6). We have recently reported a technique for separation of chemoattractant responsive and nonresponsive monocytes (7) and have shown that the nonmigrating subpopulation does not express receptors for the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP). The present study was undertaken to determine whether the isolated nonmigrating and migrating subpopulations differed in their superoxide production. Monocytes were exposed to phorbol myristate acetate (PMA), FMLP, or N-formyl-norleucyl-leucyl-phenylalanine (FNLP), and and their superoxide generation was quantified and compared with generation by unseparated monocytes.

ABBREVIATIONS: PMA, phorbol myristate acetate; FMLP, N-formyl-methionyl-leucyl-phenylalanine; FNLP, N-formyl-norleucyl-leucyl-phenylalanine; Gey's-BSA, Gey's balanced salt solution with 0.2% bovine serum albumin; PBS, phosphate buffered saline.

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MATERIALS AND METHODS

Reagents. Horse heart ferricytochrome c (Type VI), N-formyl-methionyl-leucyl-phenylalanine, N-formyl-norleucyl-leucyl-phenylalanine, bovine superoxide dismutase (Type I), phorbol myristate acetate, casein and zymosan A were purchased from Sigma Chemical Co., St. Louis, MO. Hanks and Geys balanced salt solutions and trypan blue dye solution were purchased from Grand Island Biological Co., Grand Island, N.Y.

Leukocyte isolation. Human monocytes were obtained from peripheral blood of normal donors who were leucapheresed for a 2 hr period at the National Institutes of Health Clinical Center Leucapheresis Unit, Bethesda, MD. Leukocytes were obtained with an IBM 2997 Blood Separation Channel (IBM Corp., Princeton, N.J.) maintained at a speed of 920 rpm with a flow rate of 50-60 ml of blood per min. Donors were anticoagulated with acid citrate dextrose-A and 3,000 units of heparin. Leukocyte yield at the conclusion of the 2 hr procedure ranged from 5 X 10^9-10^{10} leukocytes/collection. Leukocytes were layered on Ficoll-Hypaque and the mononuclear cells were isolated (8). Final cell preparations contained 40-60% monocytes with lymphocytes as the remainder of the cell preparation. Neutrophils represented less than 1% of the final suspension. Suspensions were prepared in Gey's balanced salt solution with 0.2% bovine serum albumin (Gey's-BSA) for chemotaxis experiments and in Hanks salt solution without protein for superoxide assays.

Separation of migrating and nonmigrating monocytes. Migrating and nonmigrating cells were isolated with a chemotaxis separation chamber described previously [(7), Neuroprobe, Inc., Bethesda, MD.]. Two ml aliquots of monocytes (4 X 10^6/ml Gey's-BSA) were placed in each upper compartment of the chamber. Lower compartments contained 1 mg/ml of casein in Gey's-BSA. Upper and lower compartments were separated by a polycarbonate membrane filter (10 um thick with 5-um pores; Nucleopore Corp., Pleasanton, Calif.). Chambers were incubated for 70 min at 37°C in moist air containing 5% CO_2. After the separation step both migrating and nonmigrating monocyte suspensions were centrifuged, washed once with Hanks and resuspended in 4°C Hanks salt solution without protein. At least 98% of the cells in both populations were viable.

Assay of superoxide production. Superoxide production was assayed by a modification of the continuous assay procedure of Cohen and Chovaniec (9). Reaction mixtures containing 2 X 10^6 monocytes in 1.0ml of Hanks salt solution with 0.1mM ferricytochrome c were preincubated for 3-5 min at 37°C before the addition of stimuli. Reference cuvettes contained the same components and 60 ug of superoxide dismutase. Data for PMA-stimulated monocytes represent initial linear rates of superoxide generation. Since monocytes displayed a burst of superoxide production similar to the kinetics described by Yasaka et al. (10), that was completed within 3 min of exposure to FMLP or FNLP, the peptide data are expressed as the total superoxide generated in 3 min. The extinction coefficient of 20,000 $M^{-1}cm^{-1}$ at 550 nm was used for cytochrome c (11).

Multiwell chemotaxis assay. Chemotaxis was quantified in a multiwell chamber described previously (12). Bottom wells contained 25 ul of attractant in Gey's-BSA, and the upper wells contained 50 ul of cell suspension (50,000 cells/well). Chambers were incubated for 70 min at 37°C. After incubation, the polycarbonate filter was removed from the chamber, nonmigrated cells were wiped off the top surface of the filter and the filter was fixed in methanol and stained with Diff-Quik (Harleco, Gibbstown, N.J.). Migrated monocytes were counted with an Optomax Image Analyzer (Optomax, Incorp., Hollis, N.H.).

Phagocytosis of opsonized zymosan. Zymosan was suspended in phosphate buffered saline (PBS), boiled for 45 min, washed and suspended in PBS at a concentration of 20 mg/ml. One ml of suspension was centrifuged, and after the

supernatant was discarded, 10 ml of human serum was added to the zymosan pellet. After incubation at 37°C for 30 min, the zymosan was washed twice with cold PBS and suspended at a concentration of 10 mg/ml (2.5 X 10^8 particles/ml). For the phagocytosis assay, 1 ml of a cell suspension containing 2 X 10^6 monocytes/ml was mixed with 80 ul of opsonized zymosan, centrifuged for 5 min at 400 X g and incubated for 25 min at 37°C. The pellets were then resuspended, centrifuged in a cytocentrifuge and stained with Diff Quik. The percentage of monocytes with at least 3 zymosan particles was determined.

RESULTS AND DISCUSSION

Chemotaxis and superoxide production of unseparated monocytes. Unseparated monocytes (monocyte preparations containing both migrating and nonmigrating subpopulations) were tested for their chemotactic responsiveness to casein and the peptides, FMLP and FNLP, and dose response curves were made for each attractant. Optimal chemotactic responses occurred to casein at a concentration of 1 mg/ml and to both peptides at 10^{-8}M. At optimal concentrations the total percentage of migrating monocytes was 20-35% of the input number, similar to previous reports from our laboratory (5,12). All three attractants were equivalent with respect to the percentage of responding monocytes.

Experiments were performed to determine which attractant could be used to separate the subpopulations without altering their metabolic responsiveness to the stimuli. Cells were suspended in medium alone or in optimal concentrations of each attractant and incubated for 2 hr at 37°C. After the

TABLE I.

SUPEROXIDE PRODUCTION BY UNSEPARATED MONOCYTES PREINCUBATED WITH CHEMOATTRACTANTS

	SUPEROXIDE PRODUCTION[2]		
PRETREATMENT[1]	PMA	FMLP	FNLP
Casein (1mg/ml)	33.2	33.3	36.7
FMLP (10^{-8}M)	36.6	0	0
FNLP (10^{-8}M)	33.3	0	0
Medium (37°C)	30.2	31.7	33.3
Medium (4°C)	30.6	27.1	21.7

[1]Monocytes (10^7/ml) were suspended in either chemoattractant or Gey's-BSA alone in polypropylene tubes for 2 hr at 37°C. After the incubation, monocytes were centrifuged, washed once and suspended in 4°C Hanks salt solution until assayed for superoxide production.

[2]Superoxide production was assayed by a continuous assay procedure (9). PMA (1ug/ml)-stimulated monocyte data are expressed as the initial linear rates of superoxide-dependent ferricytochrome c reduction (nmoles /min/ 10^7 monocytes). N-formyl peptide-stimulated monocyte data represent the total superoxide production during the first 3 min of exposure to the peptides ($5X10^{-7}$M) and are expressed as nmoles /3 min/ 10^7 monocytes.

TABLE II

PMA-INDUCED MONOCYTE SUPEROXIDE GENERATION[1]

EXPERIMENT NUMBER	UNSEPARATED MONOCYTES	NON-MIGRATING MONOCYTES	MIGRATING MONOCYTES
1	30.2	10.4	39.2
2	21.0	5.6	33.0
3	20.8	3.5	31.0
4	43.0	13.0	40.0

[1]Data represent the initial rates of superoxide-dependent ferricytochrome c reduction (nmoles /min/ 10^7 monocytes). PMA was used as the stimulus at a final concentration of 1 ug/ml.

incubation, monocytes were centrifuged, washed and resuspended in 4°C Hanks salt solution. Monocytes were then assayed for their superoxide formation to previously determined optimal superoxide-inducing concentrations of PMA (1ug/ml) and peptides (5 X 10^{-7}M). Optimal chemotactic concentrations of casein (500-1000 ug/ml) failed to stimulate monocyte superoxide production. As shown in in Table I, casein was the attractant of choice for the chemotactic separation procedure since casein-exposed and control (medium alone) monocytes responded similarly to all three metabolic stimuli. The kinetics of superoxide generation were also identical for casein-exposed and control monocytes (data not shown). Monocytes preincubated with peptides did not generate superoxide when subsequently exposed to the peptides, even though their response to PMA was normal (Table I). Since casein did not metabolically desen-

TABLE III

PEPTIDE-INDUCED MONOCYTE SUPEROXIDE GENERATION[1]

EXPERIMENT NUMBER	PEPTIDE	UNSEPARATED MONOCYTES	NON-MIGRATING MONOCYTES	MIGRATING MONOCYTES
1	FMLP	31.7	12.0	40.0
	FNLP	33.3	6.3	38.3
2	FMLP	20.0	3.5	25.8
	FNLP	15.0	3.0	15.5
3	FMLP	30.0	0	50.0
	FNLP	15.0	0	27.0
4	FMLP	67.5	21.0	90.5
	FNLP	67.5	15.0	75.0

[1]Data represent nmoles of superoxide-dependent ferricytochrome c reduction (nmoles /3min/ 10^7 monocytes). The final peptide concentration in reactions was 5 X 10^{-7}M.

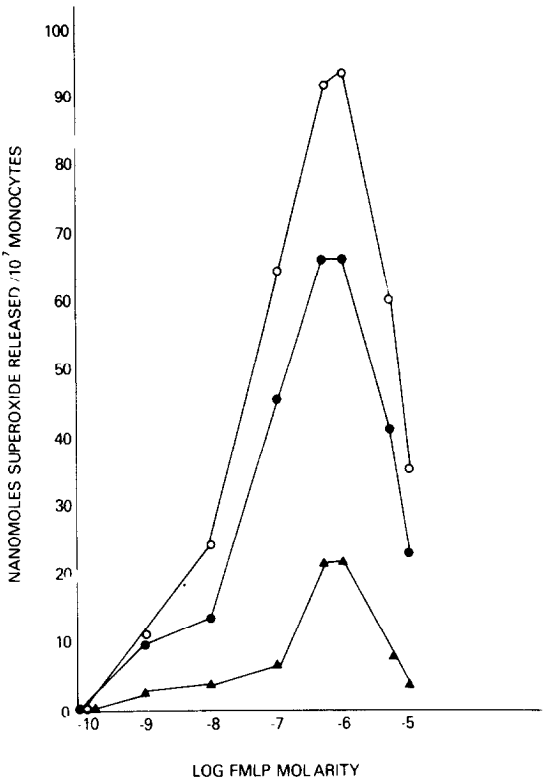

Fig. 1. Dose responses of monocyte superoxide production to FMLP. Unseparated (●), migrating (○) and nonmigrating (▲) monocytes were exposed to varied concentrations of FMLP and assayed for superoxide-dependent ferricytochrome c reduction.

sitize the monocytes, it was used as the chemoattractant in the collection chamber to separate the migrating and nonmigrating monocyte subpopulations.

Superoxide production by nonmigrating and migrating monocytes. Migrating and nonmigrating subpopulations were compared with unseparated monocytes for their PMA- and peptide-induced superoxide production. When exposed to PMA, nonmigrating monocytes had significantly depressed superoxide production which ranged from 11-33% of the activity generated by migrating monocytes (see Table II). N-Formyl peptide-induced superoxide generation by nonmigrating monocytes was also depressed, ranging from no superoxide production to 30% of the migrating population's response (see Table III). Superoxide dose responses of migrating and nonmigrating monocytes were performed for the three stimuli. The data in Figure 1 illustrate the dose responses of monocyte subpopulations to FMLP. Nonmigrating monocytes demonstrated depressed superoxide production over the en-

tire concentration range. Superoxide production by unseparated monocytes was usually less than production by an equal number of migrating monocytes. Similar differences in superoxide production were also observed when subpopulations were exposed to FNLP or PMA. Thus, the majority of superoxide activity of unseparated monocytes is generated by the chemotactically-responsive subpopulation.

The significantly depressed superoxide production by nonmigrating monocytes could not be attributed to a loss of cell viability, since at least 98% of the monocytes were trypan blue negative after the separation procedure. When incubated with serum opsonized zymosan, 98-100% of the nonmigrating and migrating monocytes internalized three or more zymosan particles per cell. Thus, the phagocytic activity and viability of nonmigrating and migrating monocytes were identical.

Recently, we have reported that the nonmigrating monocyte subpopulation does not express FMLP receptors (7). One explanation for the defective superoxide production with N-formyl peptide stimuli may be the absence or low density of FMLP receptors on the nonmigrating monocytes' membranes. The defective superoxide response to PMA may also be due to a difference in receptor expression. PMA receptors have been demonstrated on human polymorphonuclear leukocytes (13) and it is possible that they may be expressed on human monocytes. The expression of chemoattractant receptors on human leukocytes may reflect the developmental state of leukocyte. A human promyelocytic cell line, HL-60, has been differentiated in vitro and shown to develop N-formyl peptide receptors and chemotactic responsiveness to the peptides (14,15) . The nonmigrating and migrating subpopulations of the present study may be monocyte subsets in different developmental stages.

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