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Functional differences in human neutrophils isolated pre- and post-prandially

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Activated polymorphonuclear leukocytes have been associated with neoplasia, atherogenesis and reperfusion injury. Since some of these conditions are also correlated with dietary fat, we examined the functional characteristics of leukocytes isolated from subjects before and after consumption of a lipid-rich meal. There was up to 2-fold greater superoxide generation in response to agonists in leukocytes obtained post-prandially; the maximum increase was observed about 4 h after eating and followed the peak (2–4 h) in serum triglycerides. Neutrophils isolated post-prandially also exhibited impaired chemotaxis and defective bacterial killing, but normal phagocytosis. These findings provide a new variable that should be considered in studies of leukocytes.

Neutrophil; Respiratory burst; Chemotaxis; Lipoprotein; Diet

1. INTRODUCTION

Human polymorphonuclear leukocytes (neutrophils, PMN) are the first line of defense against microbial infection [1]. Produced in the bone marrow, these cells reside approximately 6 h in circulation wherein they are exposed to various circulating factors, such as serum lipoproteins [2]. They subsequently may enter tissues and in a complex series of events, locate, phagocytize and kill invading microbes [1]. Concomitant with phagocytosis, there is a marked stimulation of non-mitochondrial oxygen consumption, known as the respiratory burst [1–4]. This process is a major microbicidal mechanism of these cells and involves the production of superoxide and other highly reactive oxygen metabolites (e.g. hydrogen peroxide, hydroxyl radical, hypochlorous acid). The importance of the neutrophil in host defense is evidenced by the observation of severe and/or chronic infections associated with a number of conditions of defective neutrophil function (e.g. chronic granulomatous disease, localized juvenile periodontitis) or depressed neutrophil number (e.g. leukemias, aplastic anemias) (reviewed in [5]).

Neutrophils function not only in microbial killing, but may also, under conditions of inappropriate activation, damage host cells and their genomes. For example, neutrophil-derived oxygen metabolites are mutagenic toward *Salmonella* [6] and induce neoplastic transformation in mouse fibroblasts [7]. Neutrophils have been suggested to participate in the pathogenesis of a variety of human diseases including cancer [7–9], atherosclerosis [10–12], reperfusion injury [13], adult respiratory distress syndrome [10,14], arthritis [15], and inflammatory bowel disease [14,16].

Some of the above conditions are known to be influenced by dietary factors. For example, the correlation between a high-fat diet and development of arterosclerotic disease is well-documented [17]. In addition, epidemiological studies in humans have correlated fat intake with the incidence of cancer in breast, prostate, and large bowel [18,19]. In a rodent model system, a high-fat diet is correlated with an increased incidence of breast carcinoma [20,21]. It is thus important to determine how dietary factors influence the activation and function of neutrophils. The present investigation was undertaken to (i) study the effects of feeding on the neutrophil respiratory burst, (ii) correlate these effects with meal-induced changes in serum lipoprotein levels, and (iii) study other aspects of neutrophil function (chemotaxis and killing) in the light of these discoveries. We found that several aspects of neutrophil function are altered after eating a high fat meal.

Abbreviations: PMN, polymorphonuclear leukocyte; PMA, phorbol-12-myristate, 13-acetate; fMLP, formyl-methionyl-leucyl-phenylalanine; MEM, minimal essential medium; HBSS, Hank's balanced salts solution

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2. MATERIALS AND METHODS

2.1. *Materials*

Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from

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American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficol, 9.4% sodium diatrizoate) was purchased from Bionetics Laboratory products. Phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), cytochrome *c* (horse heart, type III), fluorescein isothiocyanate, 4',6-diamidino-2-phenylindole, propidium iodide and Acridine orange were purchased from Sigma. MEM and Hank's balanced salt solution was obtained from Gibco. Mono-poly Resolving Medium was obtained from Flow Laboratories. Histopaque 1.077 and Histopaque 1.119 were purchased from Sigma. All other reagents were of the highest quality commercially available.

2.2. *Neutrophil isolation*

Peripheral blood was obtained by venapuncture from normal donors after obtaining informed consent. Human neutrophils were isolated by sedimentation in Ficoll as previously described [22]. The cells were suspended in 2.6 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 136 mM NaCl, 8 mM Na_2HPO_4, 0.6 mM $CaCl_2$, 5.5 mM glucose (PBS-glucose) and used immediately.

2.3. *Superoxide generation*

Superoxide production was measured by continuous monitoring of cytochrome *c* reduction using an SLM/Aminco DW2000 spectrophotometer in the dual wavelength mode (A_{549}-A_{540}). The assays were performed at 37°C in stirred cuvettes using 1×10^6 in cells in 2.5 ml PBS-glucose buffer containing 50 μM cytochrome *c*. The reduction of cytochrome *c* was completely inhibited by the inclusion of 10 μg/ml superoxide dismutase in the reaction mixture. A difference extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ was used to calculate the quantity of cytochrome *c* reduced [22]. The maximal rate was calculated from the slope after an initial lag (typically around 30 s) following the addition of agonist (either PMA or fMLP, as in figure legends).

2.4. *Serum component analysis*

Serum levels of triglycerides, cholesterol and glucose were determined by a Cobas Bio-centrifugal Analyzer using certified clinical standards. Protein concentration was determined by the Bio-Rad dye-binding assay using bovine serum albumin as the standard.

2.5. *Neutrophil chemotaxis*

Chemotaxis was measured by the modified under agarose method of Chenoweth et al. [23]. Chemotaxis was assessed by monitoring the distance of migration of cells from a central well in a agarose-coated slide toward a well containing either fMLP in sterile 0.5% gelatin or the gelatin solution alone as a random migration control. Directed migration is expressed as the distance toward the chemoattractant-containing well minus that toward the control well. Data were calculated as described in the legend to Table I. A series of fMLP concentrations were initially used to obtain the optimal concentration for these conditions, and data herein are reported for 1 μM fMLP. Purified neutrophils were centrifuged and suspended in 1 × MEM (pH 7.4) and a total of 2×10^5 cells were used in each test well. The slides were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 2 h. The slides were then fixed for 8 h in methanol and cells were visualized with Wright's stain.

2.6. *Neutrophil phagocytosis and killing*

Bacteroides gingivalis (Bg) A7436 were grown in dialyzed Schandler broth in an atmosphere of 5% CO_2, 10% H_2, 85% N_2 at 37°C for approximately 72 h. A modification of the fluorochrome assay described by Cutler et al. [24] was used to monitor internalization of *Bg* by PMN. Briefly, *Bg* were grown overnight, harvested by centrifugation, resuspended in distilled water and labeled with 7.5 μg/ml diamidino-2-phenylindole for 10 min at 25°C. The bacteria were pelleted and washed 5 × with normal saline and resuspended at 5×10^7 colony forming units per ml. The bacteria were opsonized with rabbit anti-*Bg* serum at 37°C. The reaction mixture contained 25 μl serum, 25 μl saline and 75 μl bacterial suspension; the opsonization proceeded, with mixing, for 15 min at which time 100 μl of PMN (5×10^6/ml) in Hanks balanced salt solution (HBSS), 20 μl of propidium iodide (30 μg/ml) and 5 μl of 10 × HBSS (with Ca^{2+} and Mg^{2+}) were added. At 15, 30, 45 and 60 min intervals 30 μl aliquots were removed and placed in chambers in a cytospin apparatus along with 8 μl of Acridine orange (30 μl/ml) and centrifuged onto slides at 850 rpm for 3 min. Dried samples were fixed with cyanoacrylate and the slides were examined by epi-illumination UV microscopy on an Axioscope microscope. At least 100 neutrophils were scored per slide. Under UV light (due to the various fluorescent stains) live *Bg* appear blue, dead *Bg* appear red and dead PMN's nuclei stain red.

Table I

Superoxide generation in neutrophils isolated from fasted and fed donors

Agonist	Donor	Condition		% Increase
		Fasted Cyt. *c* reduction (nmol/min/10^6 cells)[a]	Fed Cyt. *c* reduction (nmol/min/10^6 cells)[a]	
fMLP (1.6 μM)	A	8.5 ± 0.1	12.9 ± 0.2[b]	52.6 ± 1.9
	B	6.4 ± 0.3	8.1 ± 0.2[c]	26.6 ± 6.4
	C	4.9 ± 0.2	8.6 ± 0.1[b]	72.2 ± 4.9
	D	11.7 ± 0.2	16.3 ± 0.3[b]	39.5 ± 4.4
	E	8.7 ± 0.3	11.2 ± 0.1[c]	28.5 ± 5.3
PMA (2.3 μM)	A	12.2 ± 0.1	17.9 ± 0.2[b]	47.3 ± 1.8
	B	12.6 ± 0.1	13.8 ± 0.0[b]	9.2 ± 0.6
	C	5.2 ± 0.2	10.1 ± 0.1[b]	89.8 ± 4.4
	D	13.6 ± 0.1	17.1 ± 0.2[b]	25.2 ± 2.0
	E	7.2 ± 0.2	9.1 ± 0.2[c]	28.0 ± 5.4

[a] Mean ± SE of 3 determinations.

[b] Means of the rates of the oxidative burst, in neutrophils isolated from overnight fasted donors vs those isolated two hours postprandially, are significantly different by Student's *t*-test for paired data $P<0.01$.

[c] $P<0.05$.

Neutrophils were obtained from normal donors after an overnight (12 h) fast and again 3 h after consumption of a typical breakfast (donors A-C) or a defined lipid-rich meal (donors D and E). The maximal rate of superoxide generation was determined by continuous monitoring of the superoxide dismutase inhibitable cytochrome *c* reduction [37], following the addition of the agonists formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA). The % increase in rate of superoxide generation, [(Fed rate/Fasted rate) − 1] × 100%, in response to 1.6 μM fMLP and 2.3 μM PMA is presented for 5 donors.

3. RESULTS

We initially observed anecdotally that neutrophils from donors who had eaten breakfast seemed to be more active in agonist-induced responses. To determine the validity of this observation, we examined the neutrophil respiratory burst of the same subjects before and after eating (Table I). Cells were obtained from donors after an overnight fast and again 3 h after eating: donors A–C consumed a typical breakfast (eggs, sausage, orange juice) while donors D and E consumed a defined lipid-rich meal (cream, milk) for purposes of comparison. The breakfast provided approximately 0.8 to 1.0 g fat/kg body mass compared to 1.2 g fat/kg for the defined meal. These values were obtained from tables defining the fat and cholesterol content of foods [25]. After eating, there was a consistent enhancement of the maximal rates of superoxide generation in response to two agonists: the maximal rate of superoxide generation, averaged for the five donors, increased 43 ± 9% and 40 ± 14% (mean ± SE) when cells were activated with fMLP and PMA, respectively. Thus, the neutrophils isolated post-prandially appear to be 'primed', that is, their response to agonists is potentiated [26]. It is not known, however, whether this is due to the priming of circulating neutrophils or mobilization of a more active subpopulation of neutrophils into the circulation.

To determine the kinetics of the priming by feeding and its correlation with changes in serum factors, we performed the experiment shown in Fig. 1. Blood was drawn at the indicated time points from the same donor either following the defined meal (filled symbols) or, on the subsequent day, with fasting (open symbols). Neutrophils were isolated from each blood sample immediately after blood was drawn. Superoxide generation in response to both fMLP (Fig. 1A) and PMA (Fig. 1B) increased with time in neutrophils from fed but not fasted individuals, and became maximal at about 4 h, remaining elevated for up to 12 h during which the subject was allowed to consume only water. For fMLP and PMA, the activities at 4 h were 106% and 60% higher than the corresponding fasted controls. No increase was seen with either agonist when the donor continued to fast over this period (Fig. 1A and B, open squares).

Three components that generally increase in circulation after eating are insulin, glucose and triglycerides. Incubation of neutrophils with low (10 μU/ml) to high (1 mU/ml) concentrations of insulin had no effect on the respiratory burst (data not shown). Furthermore, serum glucose (78.9 ± 4.4 mg/100 ml) and cholesterol (153.7 ± 1.7 mg/100 ml) did not vary significantly over this time course (i.e. samples obtained at two-hour intervals postprandially), either in the fed or fasted state and these, also, do not appear to effect neutrophil function directly. However, triglycerides increased dramatically after eating and were greatest at 2 to 4 h,

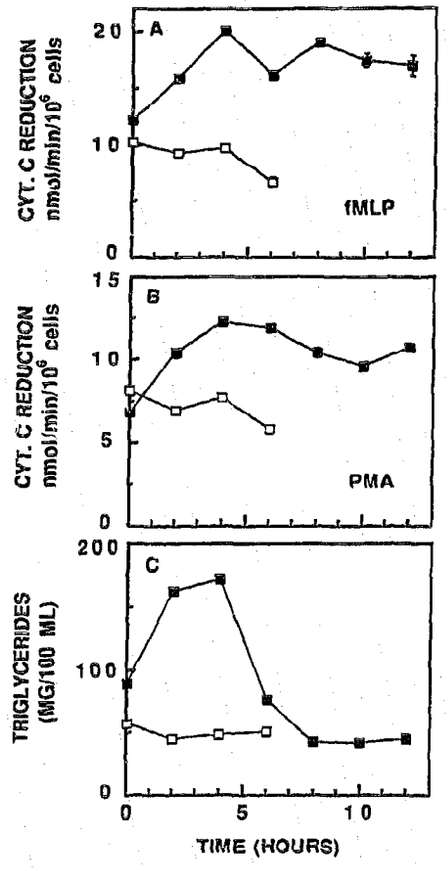

Fig. 1. Time course for augmentation of the respiratory burst and elevation of serum triglycerides after eating. Neutrophil were isolated from normal donors after an overnight fast ($t=0$) and at different times after a defined high fat meal (1.2 g fat/kg body mass) (closed squares) or after continued fasting (open squares). The maximal rates of superoxide generation elicited by 1.6 μM fMLP (panel A) and 2.3 μM PMA (panel B) are shown as the mean ± SE for three determinations (where no error bars are shown, the SE was less than the size of the symbol). Serum triglyceride levels (panel C) were determined by a Cobas Bio-centrifugal Analyzer, using certified clinical standards. Similar results were obtained with three separate donors.

after which the levels declined rapidly (Fig. 1C). Thus, it appears that the changes in neutrophil function are associated with an increase in circulating triglycerides, although the elevation in serum triglycerides slightly preceded the augmentation of the respiratory burst. Preliminary experiments involving the preincubation of neutrophils from fasted donors with chylomicrons have shown a similar augmentation of the respiratory burst (Uhlinger, unpublished).

The effects of eating on other aspects of neutrophil function were also examined. Chemotaxis towards fMLP was reduced an average of 48% in cells isolated from 3 donors, 3 h after consumption of a defined lipid-rich meal, compared with cells isolated after an overnight fast (see Table II). In separate control experiments (not shown) neutrophils were obtained both after an overnight fast and again after three hours of

Table II

Chemotactic response of neutrophils isolated from fasted and fed donors

Donor	Fasted	Fed	Fed % of fasted migration
	Distance (mm)	Distance (mm)	
X	1.54 ± .06	0.92 ± .18	60%
Y	0.52 ± .03	0.14 ± .06	27%
Z	0.56 ± .09	0.38 ± .06	68%

Neutrophils were obtained from donors after an overnight fast and again 3 h after consumption of a defined lipid-rich meal. Neutrophil chemotaxis under agarose was measured in response to 1 μM fMLP as described in section 2. The fed % of fasted neutrophil chemotaxis, (Fed migration/Fasted migration) × 100%, is presented for 3 donors. The data are the average ± range of duplicate measurements.

continued fasting; no differences in chemotaxis could be demonstrated.

Bacterial killing, but not phagocytosis, was impaired in neutrophils from fed donors. The percentage of bacteria which were killed after phagocytosis was markedly reduced in cells isolated from fed donors, in particular at early time points. This is expressed in Fig. 2A (upper panel) as the ratio of living to dead bacteria inside the neutrophil, determined by fluorescence as described in section 2. As shown in Fig. 2B (lower panel), the total number of bacteria (living plus dead) per cell which were phagocytosed were essentially identical at all time points regardless of prior feeding.

4. DISCUSSION

These studies have shown that neutrophil function is affected by eating. The respiratory burst was potentiated postprandially as shown in Table I and Fig. 1. The priming of the respiratory burst followed soon after the increase in serum triglycerides (Fig. 1), but persisted long after the serum triglyceride levels had returned to fasting levels. Neutrophils isolated postprandially also exhibit impaired chemotaxis (Table II) and diminished bacterial killing (Fig. 2A) despite normal phagocytosis (Fig. 2B). The mechanism for the diminished bactericidal capacity of these neutrophils is not certain. Although enhanced superoxide generation is seen, it may not be directed to the phagosome. Alternatively, decreased killing may relate to effects on non-oxidative killing mechanisms, including secretory responses.

Although these studies have not proven that the changes in neutrophil function were due to circulating lipids there are reasons to expect that this is the case. Neutrophils isolated from rats fed a high fat diet demonstrated both an augmented oxidative burst and decreased chemotaxis, in response to formyl peptide, relative to PMN from control animals [27]. Unsaturated fatty acids activate neutrophils [28–30], and are released from chylomicrons via the action of

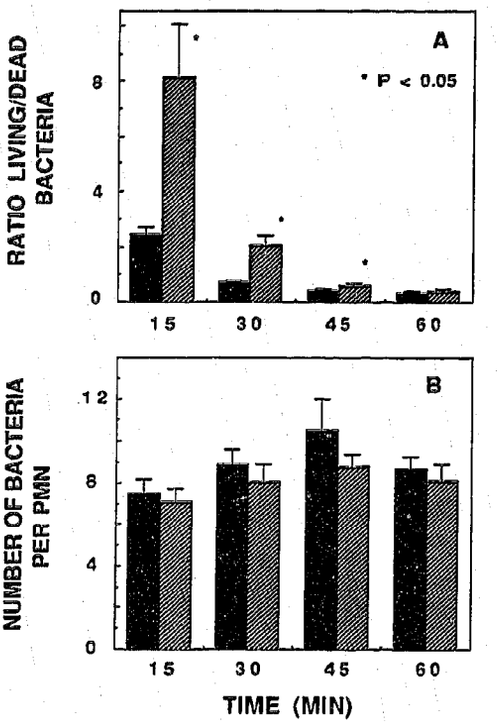

Fig. 2. Neutrophil phagocytosis and bacterial killing. PMN were isolated from donors after an overnight fast and again 3 h after consumption of a defined lipid-rich meal. The neutrophils were mixed with opsonized *Bacteroides gingivalis* and incubated at 37°C as described in section 2. The data are combined from two donors with 150 neutrophils scored per donor per time point. In the upper panel (2A) the ratio of live to dead bacteria (± SE) contained in phagocytosing neutrophils isolated from fasted donors (filled columns) is compared to that from PMN isolated 3 h postprandially (cross-hatched columns). The total number of bacteria (living + dead) per phagocytosing neutrophil ares shown in the lower panel (2B). Data are presented as mean ± SE as described above.

lipoprotein lipase. These may function via direct or indirect activation of protein kinase C, which is thought to mediate activation of the respiratory burst [22,31]. Diacylglycerols also activate [32,33] and prime [34] neutrophils, and occur as intermediates of triglyceride hydrolysis. These, however, are less likely to be involved because they are predominantly of the wrong isomeric form (i.e. 2,3- vs 1,2-diacylglycerol) for protein kinase C activation [35]. In addition, lysophospholipids are known to affect the activity of protein kinase C [36].

Whatever the mechanism, it is interesting that short-term dietary manipulation is sufficient to elicit these responses in neutrophils from normal subjects. This does not appear to be a variable that has been appreciated by biochemists, nutritionists, or others who have conducted studies using neutrophils. These findings suggest that there should be a greater effort to standardize the nutritional status of donors used as the source of human leukocytes for some types of studies.

While these alterations in PMN activity may reflect many different levels of regulation, it is tempting to

speculate that diet-related alterations in neutrophil function may have an impact on the health of individuals consuming high fat diets. On the one hand, this could relate to the ability to fight infections due to depressed neutrophil bactericidal capacity. On the other, the enhanced superoxide generation may produce or exacerbate damage to host tissues in inflammatory or other conditions, and such direct damage (e.g. to the cell itself or to its genome) may relate to carcinogenesis, atherosclerosis, etc. Neutrophil activation may have pathological consequences via effects on other components. For example, neutrophils have been shown to oxidize LDL into forms that are cytotoxic and oxidized LDL may also serve to recruit monocyte/macrophages to vascular sites which ultimately become atherosclerotic lesions [10]. Thus, the dietary effects on neutrophil function seen in the present studies may provide a mechanistic link between diseases associated with dietary fat [17-19] and neutrophil-mediated pathological processes [6-16].

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