Oxidative Metabolic Response and Microbicidal Activity of Human Milk Macrophages: Effect of Lipopolysaccharide and Muramyl Dipeptide

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Mouse macrophages can be primed by exposure in vitro to the bacterial products lipopolysaccharide and muramyl dipeptide (MDP) or in vivo by injection of MDP, so that they produce more of the bactericidal agent superoxide anion (O$_2^-$) when stimulated by phagocytosis or by contact with phorbol myristate acetate (PMA). Because little is known about the physiology of human tissue macrophages, we examined release of O$_2^-$ by milk macrophages obtained from 45 normal women for the ability to undergo priming for greater O$_2^-$ release. In samples from the same individuals, PMA-stimulated O$_2^-$ release was similar from colostrum (0 to 3 days postpartum) or from transitional milk (5 to 8 days). Release of O$_2^-$ by milk macrophages was almost identical to that by blood monocytes from the same women. Milk macrophages phagocytized and killed Candida albicans relatively effectively. Incubation with lipopolysaccharide activated the macrophages in that they were primed for greater PMA-stimulated O$_2^-$ release. Incubation with the adjuvant MDP or its analog 6-O-(2-tetradecylhexadecanoyl)-MDP did not prime, but incubation with a second analog, 6-O-(stearyl)-MDP, primed the macrophage for greater O$_2^-$ release. These results indicated that human tissue macrophages can be primed for greater oxidative response by exposure to bacterial products. Potential exists for the therapeutic use of such immunomodulating agents in the enhancement of host defense.

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MATERIALS AND METHODS

Collection of breast milk samples and preparation of cells. Milk was obtained from 45 normal lactating women with a breast pump, after informed consent was obtained. The milk was placed into sterile 50-ml siliconized tubes. Samples were generally obtained before the infants were fed. The total cell count in milk was $1.1 \times 10^6$ cells per ml (mean, $n = 20$; range, $1.5 \times 10^5$ to $5.0 \times 10^5$). Milk samples were immediately diluted 1:3 with Hanks balanced salt solution (HBSS; M. A. Bio-products, Los Angeles, Calif.) and centrifuged at 4°C and 300 x g for 10 min. The supernatant milk was recentrifuged to ensure maximum yield of cells per sample, and the pellets were combined. Cells were approximately 50% mononuclear and 50% polymorphonuclear, although this was highly variable. Cells were layered onto Ficoll-Hypaque and centrifuged at 300 x g for 30 min. The mononuclear cell layer was washed twice with HBSS and suspended at a concentration of 10$^6$ cells per ml in Dulbecco modified Eagle medium containing penicillin and streptomycin. The cells were allowed to adhere to tissue culture dishes for 1 h at 37°C. Adherent cells were washed vigorously twice with HBSS. After the adherence, cells were assayed immediately or incubated overnight in medium. Adherent cells were found to be 90 to 95% mononuclear by Wright’s stain, >90% esterase positive, and 99% phagocytic for Candida albicans (4). The preparation and culturing of cells were carried out in media and buffers shown to be free of detectable endotoxin, as tested by the Limulus amebocyte lysate assay (4).

Preparation and culture of human blood monocytes. Blood was drawn from 15 lactating women and from healthy adult donors, and 0.38% citrate was used as an anticoagulant. Monocytes were isolated as previously described (18) by using sedimentation with dextran and centrifugation in Ficoll-Hypaque. The mononuclear cell preparations con-
tain <1% contaminating granulocytes. Mononuclear cells were resuspended to a concentration of 10^6 cells per ml in Dulbecco modified Eagle medium if assays were to be performed immediately or to a concentration of 3 x 10^5 cells per ml for 7-day cultivation in Dulbecco modified Eagle medium and autologous serum. Cells were allowed to adhere for 1 h at 37°C and then washed vigorously to remove nonadherent cells before assay or further culture with stimulating agents.

Stimulating agents. The reagent lipopolysaccharide (LPS) was a gift from Floyd McIntire of the University of Colorado School of Dentistry. LPS was prepared by phenol extraction of *Escherichia coli* K-225. The extracted material was subsequently purified by gel filtration in the presence of deoxycholate (11). MDP, the inactive dD stereoisomer of MDP, and 6-O-(stearoyl)-MDP (L18-MDP) were obtained from Calbiochem-Behring (La Jolla, Calif.); 6-O-(2-tetradecylhexadecanoyl)-MDP (B30-MDP) was generously given to us by S. Kotani, Osaka University. Stimulating agents were incubated overnight with cells in medium.

Determination of cell number. Milk and blood mononuclear cells adherent to culture dishes were lysed with Zap-O-globin II (Coulter Electronics, Inc., Hialeah, Fla.). The residue was scraped off the dish with a rubber policeman and diluted 1:20 in HBSS, and nuclei were counted with a Coulter Counter model ZB (Coulter). In some experiments, nuclei counts were confirmed by counting with a hemacytometer.

O₂⁻ release. Adherent cells were washed twice with HBSS and then assayed for production of O₂⁻ measured as superoxide dismutase-inhibitable reduction of ferricytochrome c (6, 7). The stimulus was PMA (0.5 μg/ml; Consolidated Midland Corp., Brewster, N.Y.). After removal of the O₂⁻ assay mixture, the cells were washed three times with HBSS, and protein was determined by the Lowry method (6).

Phagocytosis of *C. albicans*. *C. albicans* (ATCC 18804) was used for assays of phagocytosis and phagocytic killing as previously described (4, 24), using cells adherent to glass cover slips and ratios of 10 candidas to 1 cell. After incubation for 60 min at 37°C, cover slips were washed and then double-stained with Wright's and Giemsa stains. Approximately 95% of cell-associated candidas could be seen within a detectable phagocytic vacuole (4).

**RESULTS**

The release of O₂⁻ was compared with macrophages from milk obtained in the first 72 h postpartum (colostrum) and from milk obtained 5 to 8 days postpartum (transitional milk) (Fig. 1). There was no significant difference in O₂⁻ production by macrophages from colostral or transitional milk in the absence of an added stimulus or when cells were stimulated with PMA (0.5 μg/ml).

Figure 2 compares O₂⁻ release by blood monocytes from lactating women to that of milk macrophages obtained concurrently. Release of O₂⁻ in the absence of an added stimulus was slightly higher in milk macrophages (*P* < 0.01, Student's *t* test), possibly because of exposure of milk cells to contaminating bacteria, but O₂⁻ release during stimulation by PMA was equivalent in the two cell types.

Macrophages obtained from inflammatory exudates or from *M. bovis* BCG-infected mice are primed to release greater amounts of O₂⁻ when stimulated with PMA than are normal, resident cells (6). In addition, mouse peritoneal macrophages can be similarly primed by exposure in vitro to the bacterial product LPS or to the synthetic glycopeptide MDP (4, 19, 24). When human milk macrophages were incubated overnight with LPS (10 ng/ml), the cells showed significant spreading, with formation of one or more pseudopods (Fig. 3), a characteristic associated with activation of macrophages (4, 9, 19). These cells also displayed a
twofold increase in PMA-stimulated $O_2^-$ release at 24 and 48 h in cultures (Fig. 4). Although mouse peritoneal macrophages can be primed for greater $O_2^-$ release when preincubated with MDP at 10 µg/ml (19), when human breast milk macrophages were incubated with MDP at this concentration, there was no increase in $O_2^-$ generation (Fig. 4). When higher concentrations of MDP, up to 100 µg/ml, were cultured with human milk macrophages overnight (Fig. 5), a slight, though insignificant, increase in $O_2^-$ release was observed ($P = 0.1$, Student's $t$ test). B30-MDP and L18-MDP, which contain long-chain fatty acids attached to muramic acid, were tested because lipophilic derivatives of MDP have been shown to be more active than MDP in priming mouse macrophages to release $O_2^-$ (17). Compared with MDP, B30-MDP has greater adjuvanticity yet weaker protective activity against sepsis in mice (10). When incubated overnight with human milk macrophages, B30-MDP (10 µg/ml) gave a slight, but insignificant, increase in $O_2^-$ (Fig. 5). In contrast, L18-MDP, which has a greater protective activity against sepsis in mice than does MDP (10), primed milk macrophages to produce twice as much $O_2^-$ as untreated cells ($P < 0.01$, Student's $t$ test).

We compared the capacity of adherent milk macrophages cultured overnight with MDP (100 µg/ml), B30-MDP (10 µg/ml), L18-MDP (10 µg/ml), or medium alone to ingest and kill *C. albicans* in four experiments. Under the conditions used, phagocytosis of candida was rapid and effective, with 99% of the fungi being ingested by 60 min after addition to adherent macrophages. Each of the four groups of milk macrophages killed approximately 16% of the initial candida inoculum at both 1.5 and 3 h of incubation. (This extent of candida killing approximates that achieved by mouse peritoneal macrophages [24].) Thus, the enhanced release of $O_2^-$ by macrophages treated with L18-MDP was not associated with enhanced candidacidal activity. Freshly isolated blood monocytes of lactating women killed 27 ± 12% (mean ± standard error of the mean; $n$, 4) of an initial candida inoculum, a result similar to that with control monocytes (30 ± 10%; $n$, 20). Control monocytes cultured for 7 days (monocyte-derived macrophages) killed less of an initial candida inoculum (20 ± 7%; $n$, 5) than when freshly isolated, and the extent of killing approximated that of milk macrophages.

**DISCUSSION**

We found that human breast milk macrophages undergo a vigorous oxidative metabolic response after stimulation. This oxidative response was equivalent in cells from colostrum and milk, and was as vigorous as the oxidative response in blood monocytes obtained concurrently from the same individual.

Our data indicate that human milk macrophages, like human blood monocytes (18) or rodent peritoneal macrophages (8, 19), have the capacity to be primed by exposure to bacterial products for a greater release of $O_2^-$ during stimulation. We have previously shown that human peritoneal...
macrophages obtained by saline lavage from trauma victims undergo a vigorous oxidative response to PMA (16). However, we are not aware of previous demonstration that human tissue macrophages can be primed pharmacologically for a greater oxidative metabolic response.

Although release of $O_2^-$ was increased by exposure of macrophages to L18-MDP, killing of $C. albicans$ was not enhanced by such exposure. We have previously shown that injection of MDP into mice primes their macrophages for a fivefold greater oxidative response and for clearly enhanced oxidative response after exposure to the MDP (19). We have also reported that killing of $C. albicans$ by mouse macrophages depends at least in part on oxidative metabolism (24). Thus, it is not clear why L18-MDP did not increase milk macrophage candidacidal activity. Oxidative metabolism measured by the chemiluminescence response of milk cells has been shown to be closely correlated to the killing of $C. albicans$ by mouse macrophages (22). The possibility was raised that quenching was only observed extracellularly and because the enhancement was twofold, it is possible that the high intracellular fat accumulation in the milk macrophages scavenged any additional metabolites induced by the MDP. Perhaps related to our findings is the observation that milk macrophages, in contrast to blood monocytes, did not show augmentation of tumoricidal activity when exposed to lipopolysaccharides (1). The capacities of macrophages to secrete oxygen metabolites and to kill microorganisms have been closely correlated for several intracellular pathogens (4, 14, 24, 26); however, oxidatively deficient human macrophages and fibroblasts can also successfully inhibit the intracellular replication of Toxoplasma gondii and Chlamydia psittaci (13), demonstrating that killing involves more than generation of oxygen radicals.

All types of immunologically active inflammatory cells pass from the human lactating mammary gland into colostrum and milk. After an initial rapid decline of cellular components during the first five days postpartum, the cellular content of breast milk remains relatively constant for at least two months (21). The milk macrophage, the most numerous of the cells of breast milk, may be responsible, at least in part, for the protection afforded by breast milk against necrotizing enterocolitis in animals (20). Reported functional capabilities of the human milk macrophages include release of lysozyme, lactoferrin, and PGF$_2$ (2), synthesis of C3 (12), secretion of epithelial cell growth-stimulatory factor (3), and accessory function in lymphocyte transformation (22). We demonstrated the following further functional activities of these phagocytic cells: vigorous oxidative response when stimulated with the surface active agent PMA and phagocytosis and killing of $C. albicans$.

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