RESEARCH ARTICLE

Lactic acid stimulates interleukin-23 production by peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide

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Received 29 June 2010; revised 18 August 2010; accepted 19 October 2010.
Final version published online 30 November 2010.
DOI:10.1111/j.1574-695X.2010.00757.x

Editor: Patrick Brennan

Keywords
lactic acid; interleukin-23; lipopolysaccharide; immune response; vagina.

Abstract

Lactic acid is the predominant acid present in the vagina. We evaluated the consequences of lactic acid, at physiological levels present in the vagina, on cytokine responses of peripheral blood mononuclear cells (PBMCs) obtained from 10 individuals in the presence or absence of bacterial lipopolysaccharide. Pre-incubation of PBMCs in 15 mM lactic acid before the addition of lipopolysaccharide resulted in a 246% mean increase in interleukin-23 (IL-23) secretion over that released in the presence of lipopolysaccharide alone (P = 0.0068). The lipopolysaccharide-induced production of tumor necrosis factor-α, IL-6, IL-10 and IL-12 was unaffected by lactic acid. IL-23 stimulation was not observed if the lactic acid was neutralized before its addition to the culture medium or if hydrochloric acid was substituted for lactic acid. In the absence of lipopolysaccharide, lactic acid did not stimulate the production of IL-23 or any of the other cytokines. The increase in IL-23 production was proportional to the lactic acid concentration over a 15–60 mM range. We conclude that at body sites characterized by lactic acid accumulation, such as in the human vagina, exposure to gram-negative bacteria results in selective IL-23 production, leading to a subsequent preferential stimulation of the Th17 T lymphocyte pathway.

Introduction

Under normal physiological conditions, the body site characterized by the continual accumulation of lactic acid is the vaginal lumen of reproductive-age women (Huggins & Preti, 1976). Lactic acid in vaginal secretions originates from the activity of both the vaginal mucosa (Gorodeski et al., 2005) and the action of Lactobacillus sp. and possibly also by other bacterial species (Zhou et al., 2004). Glucose in the intermediate vaginal epithelial cell layer under the influence of estrogen is metabolized under anaerobic conditions to pyruvic acid and then to lactic acid. The lactic acid diffuses out of the cells and accumulates in the extracellular fluid. Similarly, Lactobacillus sp. convert extracellular glucose into lactic acid by anaerobic glycolysis.

The activation of polymorphonuclear leukocytes and monocytes/macrophages is an energy-dependent process and stimulates the induction of glycolysis. Thus, inflammation is also associated with localized lactic acid release (Haji-Michael et al., 1999). Similarly, lactic acid is produced and released into the extracellular environment by many malignant tumors due to both accelerated aerobic glycolysis (the Warburg effect) (Warburg, 1961) and by anaerobic hypoxia-driven glycolysis (Elson et al., 2000).

The consequence of lactic acid release on immune system activities has not received much research attention. In a series of elegant experiments, Shime et al. (2008) demonstrated that a human lung adenocarcinoma cell line (CADO-LC10 cells) secreted lactic acid into the culture medium. While the lactic acid released by itself had no effect on cytokine induction, in the concomitant presence of a Toll-like receptor (TLR) ligand, lactic acid stimulated the production of interleukin-23 (IL-23) by monocytes/macrophages. Conversely, there was no effect of lactic acid on TLR-stimulated IL-12 transcription. IL-12 and IL-23 are heterodimeric cytokines that share a p40 subunit. In IL-12, p40 combines with a p35 subunit; in IL-23, p40 combines with p19 (Langrish et al., 2004). Thus, lactic acid enhanced p40 and p19 transcription drastically. The stimulation of IL-23 production required the presence of a lactate ion in its
transportable form; the neutralized lactate anion or the presence of an equivalent proton concentration from a different acid did not enhance IL-23 release (Shime et al., 2008).

IL-23 and IL-12 have unique effects on T helper lymphocyte subsets. IL-12 induces T cell differentiation into the Th1 CD4\(^+\) T cell subset. The release of interferon-\(\gamma\) (IFN-\(\gamma\)) by Th1 cells and natural killer cells activates macrophages to destroy intracellular microbial pathogens (Goriely et al., 2008). IFN-\(\gamma\) also acts on B lymphocytes to inhibit the synthesis of immunoglobulin G1 antibodies (Manetti et al., 1993). In contrast, IL-23 promotes the development of the newly recognized Th17 CD4\(^+\) T cell subset (Bettelli et al., 2007). Th17 cells are essential for the recruitment, activation and migration of neutrophils and the destruction of extracellular microorganisms (Vanden Eijnden et al., 2005).

The influence of lactic acid on cytokine production by peripheral blood mononuclear cells (PBMCs) has not been determined previously, and is the subject of this communication. The findings have biological relevance for an enhanced understanding of infection-related immune mechanisms operative in the lactic acid-dominated female lower genital tract.

**Materials and methods**

**Cell cultures**

Venous blood was obtained from 10 healthy female and male volunteers and PBMCs isolated by Ficoll-Hypaque (GE Healthcare Biosciences, Piscataway, NJ) gradient centrifugation. The mononuclear cell band was recovered, the cells were washed twice in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA) and resuspended in RPMI to a final viable concentration of \(1 \times 10^6\) cells mL\(^{-1}\). Viability was determined by trypan blue exclusion. The PBMCs were added to the wells of a sterile microtiter plate (\(1 \times 10^6\) cells per well) that contained RPMI medium ± various concentrations of \(\alpha\)-lactic acid (Sigma-Aldrich, St. Louis, MO) or \(\alpha\)-lactic acid that had been neutralized with sodium hydroxide to the pH of RPMI medium. In other experiments, hydrochloric acid (HCl) was added to RPMI medium to match the pH obtained by lactic acid addition. After incubation for 24 h in a 37 °C, 5% CO\(_2\) incubator, either lipopolysaccharide (50 ng mL\(^{-1}\) Escherichia coli serotype 0111:B4, Sigma-Aldrich) or an equivalent volume of RPMI was added to quadruplicate wells and incubation was continued for another 24 h. The culture supernatants were then collected by centrifugation and stored at -80 °C until assayed for cytokines. Cell viability as well as the pH in each well were checked at the conclusion of the experiment. All reagents were filter sterilized before use and a sterile technique was used throughout.

The study was approved by the institutional review board of the Weill Cornell Medical Center–New York Presbyterian Hospital and written informed consent was obtained from all participants.

**Cytokine analyses**

The culture supernatants were tested in duplicate for IL-23, IL-12, IL-10, IL-6 and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) using commercial enzyme-linked immunosorbent assay kits (ebioscience, San Diego, CA for IL-23 and IL-12; Invitrogen for IL-10 and TNF-\(\alpha\); R&D Systems, Minneapolis, MN for IL-6). Experimental values were averaged and converted to pg mL\(^{-1}\) by reference to a standard curve that was generated in parallel to the test samples. The lower limits of sensitivity were 15 pg mL\(^{-1}\) for IL-23, 4 pg mL\(^{-1}\) for IL-12, 0.2 pg mL\(^{-1}\) for IL-10, 9.4 pg mL\(^{-1}\) for IL-6 and 1.7 pg mL\(^{-1}\) for TNF-\(\alpha\).

**Statistical analysis**

The associations between cytokine levels and incubation condition were analyzed using the Mann–Whitney test. A \(P\) value of < 0.05 was considered significant. GRAPH PAD INSTAT (Graft Pad Software, San Diego, CA) was utilized for the analysis.

**Results**

**Lactic acid- and lipopolysaccharide-induced cytokine production**

The addition of lactic acid to PBMCs incubated with lipopolysaccharide resulted in a marked increase in IL-23 secretion over that released in the presence of lipopolysaccharide alone \((P = 0.0068)\). This effect was abolished if the lactic acid was neutralized before its addition to the culture medium. In marked contrast, lactic acid had no effect on lipopolysaccharide-induced TNF-\(\alpha\), IL-6, IL-10 or IL-12 cytokine release by PBMCs. These results are summarized in Table 1. Evaluating the individual results from each of the 10 subjects revealed that inclusion of lactic acid resulted in a mean 246% increase in IL-23 release over that of lipopolysaccharide alone. In contrast, IL-23 production in the presence of neutralized lactic acid was a mean of 98% of that observed with lipopolysaccharide alone (Fig. 1). In the absence of lipopolysaccharide, lactic acid did not stimulate the production of IL-23 or any of the other cytokines above background levels. Similarly, the substitution of HCl for lactic acid did not result in the stimulation of cytokine release (data not shown). Preincubation in lactic acid had no observable effect on cell viability. The gender of the PBMC donor did not influence the results.

The effect of lactic acid concentration on lipopolysaccharide-induced IL-23 production is shown in Fig. 2. IL-23

\[IL-23 = \frac{a}{b} \times c + d\]
levels increased in direct proportion to the lactic acid concentration from 15 to 60 mM and then markedly decreased at 120 mM lactic acid. The pH of the culture medium (8.0 in the absence of lactic acid) decreased to 7.5, 7.2, 7.0, 6.8 and 6.4 with the addition of 15, 30, 45, 60 and 120 mM lactic acid, respectively.

**Discussion**

Lactic acid, in a dose-dependent manner, selectively promoted the release of IL-23 by PBMCs in response to lipopolysaccharide. IL-23 maintains T helper cell development along the Th17 pathway. Th17 cells release IL-17, which induces the mobilization, recruitment and activation of neutrophils to mucosal surfaces (Kolls & Linden, 2004). In addition, proinflammatory cytokines and chemokines are induced from epithelial cells, endothelial cells and macrophages (Weaver et al., 2007). Thus, at body sites characterized by the production and release of lactic acid, contact of gram-negative bacteria with antigen-presenting cells would result in the selective activation of the Th17 T lymphocyte pathway and enhanced protection against extracellular pathogens. Lactic acid, at a concentration as low as 5 mM, has also been reported to inhibit the release of TNF-α by lipopolysaccharide-stimulated human monocytes without affecting viability (Dietl et al., 2010). However, in the present study, lactic acid did not influence TNF-α production by PBMCs. Possibly, the additional presence of lymphocytes attenuated this inhibitory activity.

The uptake of the lactate anion into cells is facilitated by a low extracellular pH, due to the formation of a pH gradient between the extracellular and the internal cellular milieu (Loike et al., 1993). Thus, the acidic environment of the human lower genital tract would be a preferred site for this activity. Lactate ion transport is an active process and requires the presence of a specific proton-linked monocarboxylate transport (MCT) system (Halestrap & Price, 1999). This system has been identified in monocytes, lymphocytes and granulocytes (Merezhinskaya et al., 2004). The only report of MCT-mediated uptake of lactic acid by female genital tract cells was in the human cervical adenocarcinoma cell line, HeLa (Cheeti & Lee, 2010). The total lactate concentration in the vagina is between 10 and 50 mM in nonpregnant women (Boskey et al., 2001) and approximately 32 mM during pregnancy (Liston & Chisholm, 2011).
1947). Thus, the lactic acid levels used in our study were within the normal physiological range for this site.

The precise mechanism of lactic acid-dependent stimulation of infection-induced IL-23 production and its consequences, in the vagina as well as at other lactic acid-producing locations, remain to be determined. An earlier study demonstrated that sodium lactate activated the nuclear factor-κB and mitogen-activated protein kinase signaling pathways in a macrophage cell line (Nareika et al., 2005). It is interesting to point out that the invasive and pathogenic hyphal form of the dimorphic fungus, Candida albicans, has been shown to selectively trigger IL-23 production (Acosta-Rodriguez et al., 2007). This results in the induction of a preferential Th17 lymphocyte response to this microorganism. The subsequent recruitment and activation of neutrophils facilitates hyphal killing (Urban et al., 2006). It has been speculated that the predominance of a Th17 memory cell response against C. albicans may be related to the environment in which the initial immune sensitization occurred (Acosta-Rodriguez et al., 2007). Because approximately 75% of premenopausal women will experience at least one episode of C. albicans vaginitis (Sobel, 1997), immune system contact to this organism typically occurs in many women in a lactic acid-dominated environment. This favors a selective exposure of C. albicans to Th17 cells. Even if lactic acid does not directly enhance IL-23 production in the presence of C. albicans, the simultaneous occurrence of multiple bacterial species in the vagina would result in IL-23 stimulation and ensure continued contact of Th17 cells with C. albicans. This might explain the preferential presence of anti-C. albicans Th17 memory cells.

Our reported influence of a lactic acid-dominated environment on immune responses to microbial pathogens should also serve as a caution to the interpretation of studies that evaluated the immune repertoire to vaginal microorganisms such as C. albicans, bacterial vaginosis-related bacteria and sexually transmitted microorganisms in an in vitro system. The exclusion of lactic acid, as well as possibly other vaginal compounds, from the experimental protocol might have led to results that were of limited relevance to the true in vivo situation. Similarly, the vaginal pH of laboratory mice, rats and rabbits is between 6.5 and 7.5 (Kaminsky & Willigan, 1982; Meysick & Garber, 1992) and devoid of Lactobacillus, substantially different from that of women. This calls into question the applicability to the human situation of studies performed on the lower genital tract in animal models. In addition, the observed failure of HCl to substitute for lactic acid suggests the specificity of lactic acid, and not just an acidic pH, for IL-23 induction. Thus, experimental protocols as well as commercial products that attempt to acidify the vagina with acids other than lactic acid do not mimic the natural environment and may be less than ideal.

The implication that lactic acid may specifically aid in immune defense leads one to question currently held beliefs about vaginal health. Vaginal lactic acid production by both the underlying epithelium (Gross, 1961) and endogenous lactobacilli and other bacteria contribute to the final lactic acid concentration. Individual differences in colonizing lactobacilli and other components of the vaginal flora, variations in the genetic background that influence glucose metabolism and unique environmental and dietary exposures would all be expected to result in variations in lactic acid production. We postulate that the extent of lactic acid production, and not bacterial hydrogen peroxide production, is a key component of the innate immune defense mechanisms at this site. A recent investigation using gene amplification technology has revealed that the major Lactobacillus sp. in asymptomatic North American women is Lactobacillus inners, a bacterium that does not produce hydrogen peroxide (Ravel et al., 2010). Another study has demonstrated that both cervicovaginal fluid and semen block any hydrogen peroxide-induced microbicidal activity (O’Hanlon et al., 2010). Further study of larger numbers of women is clearly warranted to confirm our findings as well as to help unravel the misconceptions that now exist about vaginal bacterial flora and innate defense mechanisms at this anatomical site. It would also be of interest to determine whether other organic acids that are structurally related to lactic acid, and that may be present in the vagina, have similar immunological effects. In this regard, it has been demonstrated that lactate, but not butyrate, acetate, dichloroacetate, citrate or malate, augments lipopolysaccharide-induced IL-2 production by murine splenic T cells (Roth & Droge, 1991).

In females before puberty and after menopause, vaginal lactic acid levels are much reduced and vaginal pH is elevated. Whether this contributes to a possible increased susceptibility to gram-negative bacterial infections under these conditions is not known and is worthy of investigation.

In general, mucosal infection favors the induction of the Th17 subset while intravenous infection is characterized by the induction of Th1 cells (Pepper et al., 2010). This suggests that antimicrobial immunity at mucosal surfaces is preferentially geared towards IL-23 and IL-17 induction and away from the production of Th1 lymphocyte-generated IFN-γ. The facilitation of IL-23 secretion by lactic acid would serve to further ensure the preferential activation of Th17 cells in the female genital tract even in the presence of diverse bacterial species that are normally present at that site. The induction of IFN-γ synthesis in the female genital tract is necessary for the induction of an immune response, and subsequent sensitization, of the female to spermatozoa (Witkin, 1988). It is intriguing to speculate whether perhaps an additional function of lactic acid downmodulation of Th1 cell formation in the vagina may be to help preserve
fertility by limiting an IFN-γ response to commensal bacteria and to microorganisms transmitted in the male ejaculate.

Authors’ contributions
S.S.W. designed the study, analyzed the data and prepared the original manuscript. S.A. and A.M.B. performed the experiments and collected data. I.M.L., W.J.L. and A.M.B. participated in data analysis. W.J.L. and I.M.L. participated in the final manuscript preparation. All of the authors read and approved the final manuscript.

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