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Vaginal Probiotics and *In vitro* Inhibition of Herpes Vaginal Infection

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The protective effect of selected vaginal Lactobacillus strains (*L. brevis CD2, L. salivarius FV2, L. plantarum FV9*) towards herpes simplex virus type 2 (HSV-2) infection in vitro has been analyzed. Living bacterial cells affect different steps of virus multiplication. The effect on the early phases of virus infection appeared related to the bacterial adhesivepotential to the cell membrane while all the strains strongly reduced intracellular events of virus multiplication. The antiHSV-2 activity was not mediated by a virucidal effect. Instead it was exerted through bacterial soluble factors able to down regulate the production of infective virions. In fact HSV-2 yield was significantly reduced in infected cells fed with cell-free supernatants of lactobacilli grown in cell culture medium. Purified lactic acid and H_2O_2, *Lactobacillus* metabolites with known antimicrobial activity, produced a dose-dependent virucidal effect. Lactic acid successfully interfered with viral intracellular antigen synthesis and both the virucidal activity and the inhibition of replication were correlated to acidic pH values. *L. brevis* CD2, the most active strain, does not produce H_2O_2 and neutralized lactic acid had no effect, thus indicating that factors other than H_2O_2 and lactic acid could be responsible for the antiviral effect.

Key words: Herpes simplex virus type 2, Vaginal lactobacilli.

The cervico-vaginal mucosa represents a portal of entry for different pathogenic microorganisms in women. In healthy women of child-bearing age, the protective mucosa in the vagina is populated with microflora typically dominated by lactobacilli and their dominance over pathogenic anaerobes is positively associated with vaginal health[1]. The most common vaginal disorder among reproductive age women involving a strong reduction in the number of vaginal lactobacilli is bacterial vaginosis (BV). BVis not caused by one specific pathogenic microorganism, but rather by an imbalance of the vaginalmicrobial flora. In BV, lactobacilli are reduced or absent or lacking specific antimicrobial properties (i.e. production of H2O2) and are replaced by *Gardnerella vaginalis* and other anaerobic microbiota.

Increasing data now indicate that abnormal vaginal flora lacking lactobacilli facilitates the acquisition of viral sexually transmitted diseases. The first clinical studies suggesting an association between BVand a viral sexually transmitted infection were reported for HIV. HIV seropositivity was significantly correlated with BV, independently of other behavioral variables[2, 3]. More recent prospective studies demonstrated an association between alterations of vaginal flora and acquisition of HIVinfection[1, 4]. Lack of a Lactobacillus-predominant vaginal flora was identified as a risk factor for herpes simplex

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virus type 2 (HSV-2) and human papillomavirus (HPV) infections[5, 6]. In addition, acquisition of HSV-2 and HPVinfections have both been associated with BV[6, 7]. Recently abnormal vaginal flora has been identified as a risk factor for genital tract shedding of cytomegalovirus and HSV-2 in women[8, 9]. Moreover, female genital-tract HIV load correlates inversely with vaginal lactobacilli bacterial counts[10]. Therefore lactobacilli exert in vivo is an important role in the epidemiology of sexually transmittedviral infections both in relation to the protection of female health as well as by reducing the risk of virus transmission from an infected woman to a healthy man. In spite of the protective effect of vaginal lactobacilli as indicated by the epidemiological studies, the antiviral activity of probiotic bacteria has not yet been studied in detail in cell cultures. Klebanoff and Coon demonstrated that hydrogen peroxide produced by a strain of L. acidophilus displays virucidal effect on HIV-1, particularly in the presence ofperoxidase and chloride[11]. Recently it has been reported that the infectivity of vesicular stomatitis virus was reduced after preincubation with different Lactobacillus strains[12] and that cell-free filtrates of two Lactobacilluscultures inhibited the replication of HSV-2[13]. Therefore the mechanism of action of lactobacilli towards viral infections is still poorly understood. Possible mechanisms to account for the protection exerted by vaginal lactobacilli include inactivation of pathogens by different metabolic Lactobacillus products (lactic acid, H2O2, bacteriocins), competition for epithelial cell attachment sites,preservation of mucin gel coating the vaginal/cervical epithelium through inhibition of glycosidase-producing anaerobes, and maintenance of appropriate innate immune response[14-16].

The purpose of this research was to evaluate the protective activity of vaginal lactobacilli towards HSV-2 infection in cell culture and to identify the possible mechanism of action. Worldwide, HSV-2, the primary cause of genital herpes, is one of the most prevalent sexually transmitted infections. Infection is considered life-long, as the virus becomes latent in sacral nerve ganglia, and may result in recurrent genital lesions. Genital herpes plays a major role in increasing the risk for sexual acquisition and transmission of HIV (17) and HSV-2 infection cannot be prevented by a vaccine. Three species of vaginal lactobacilli (*Lactobacillus brevis, Lactobacillus salivarius and Lactobacillus plantarum*) with different biochemical characteristics and adhesion capacity to cells have been compared for their antiviral activity. The strains have been characterized and selected for the prophylaxis and treatment of vaginal infections[18]. *L. salivarius* and *L. plantarum* strains produce anti-infective agents including hydrogen peroxide. *L. plantarumand L. brevis*strains are able to adhere at high levels to human epithelial cells displacing vaginal pathogens[19]. All the strains were able to temporarily colonize the human vagina after a 5 day-treatment[20].

MATERIALS AND METHODS

Bacterial strains and growth conditions Lactobacillus brevis (strain CD2), *Lactobacillus salivarius*subsp.salicinius (strain FV2), *Lactobacillus plantarum* (strain FV9) were stored as a stock culture at -70°C in 90% de ManRogosa-Sharpe (MRS) broth (Oxoid) and 10% glycerol. Lactobacilli were inoculated from frozen vials onto MRS broth and cultured overnight at 37°C under anaerobic conditions in Anaerogen system (Oxoid). Correlation between optical density and colony forming units Lactobacilli were thawed-out directly performing 10-fold dilutions in MRS broth up to 10 -6 to obtain mid-logarithmic-phase organisms after overnight incubation. One ml of the bacterial suspensions was washed twice in Phosphate Buffered Saline (PBS, pH 7.2) at 5000 g, 4°C, for 10 min and the optical density assessed by spectrophotometry at 600 nm. Viable microorganisms were determined by plating serial 10-fold dilutions of lactobacilli onto MRS-agar plates. Tests were performed in triplicate. Colony counts were carried out after 48 hrs incubation. Correlation between optical density and CFU was established.

Cells

Vero African green monkey kidney cells were cultured at 37°C in a 5% CO2 atmosphere in Eagle's Minimum Essential Medium (MEM, HyClone) containing 1.2mg/ml NaHCO3 and supplemented with 6% (v/v) fetal bovine serum, 2 mM glutamine, 100IU/ml penicillin and 100 µg/ml streptomycin.

For cell maintenance the serum concentration was lowered to2% (maintenance MEM). Bacterial culture supernatant (CS) preparation exponentially growing cultures of lactobacilli in MRS broth were washed thrice with PBS and re-suspended in cell maintenance MEM without antibiotics. LactobacillusCSs wereobtained growing bacteria in six-well plates in the presence or absence of Vero cells in maintenance medium without antibiotics for 16 h at 37°C in 5% CO2atmosphere. Bacteria were removed by centrifugation at 5000 g for 10 min and supernatants immediately used in the antiviral assays.

HSV-2 and viral plaque assay

The P1 strain of HSV-2, a clinical isolate from the parsian Virology Laboratory, was grown on Vero cells in maintenance medium. Sub confluent cell monolayers were inoculated with virus at a multiplicity of infection of 0.1 PFU/cell and incubated at 37°C for 48 h. After three cycles of freezing and thawing, thecultures were centrifuged at 1000 g for 20 min (4°C) to remove cellular debris, and clarified upper natants stored at -80°C. Virus titre was determined by a standard plaque assay. Serial ten-fold dilutions of virus in maintenance medium were inoculated on confluent Vero cell monolayers in 6-well plates. After a 1 hrs adsorption period at 37°C, the inoculum was removed and cells were washed three times with PBS before being overlaid with MEM containing 0.4% (w/v) agar (Oxoid). After 3 days incubation at 37°C, plaques were stained with 0.1% crystal violet solution.

Immunofluorescence assay

Vero cells were grown in micro-tissue chamber slides at a concentration of 4×10 4cells/ well for 24 hrs in 5% CO2at 37°C. Cell monolayers were infected with 1 PFU/cell of HSV-2 for 1 h at 37°C. After incubation for 16 hrs at 37°C, the percentage of infected cells was determined by a direct immunofluorescenceassay using FITC-conjugated rabbit anti-herpes simplex virus type 2 immunoglobulins (Dako). The antibody reacts with all the major glycoproteins present in the viral envelope and at least one core protein as determined by crossed immunoelectrophoresis.

Virucidal effect

Living bacterial cells, bacterial culture supernatants, H2O2 and lactic acid were incubated with HSV-2 (9×10 6 PFU/ml) for 1 h or 16 hrs at 37°C in sterile screw-cap microtubes. The tubes were centrifuged at 5000 g for 10 min to sediment bacteria where necessary and residual virus infectivity was determined by plaque assay.

Virus yield reduction assay

For antiviral assays, confluent monolayers of Vero cells in 24-well plates were inoculated with HSV-2 (1 PFU/cell). The infection was synchronized by allowing the virus to adsorb to the cells in the cold (4°C). After 1 h, the inoculum was removed by washing thrice with PBS. Then, the temperature was raised to37°C to permit internalization. Single-cycle conditions were achieved by incubating the cells at 37°C for 16 hrs post infection. The cultures were freeze-thawed three times, cell debris removed by low speed centrifugation and supernatants titrated by plaque assay on Vero cell monolayers.

Determination of H2O2production

The measurement of H2O2produced by Lactobacillusstrains was an adaptation of the technique described by Pick and Mizel[21]. Briefly, Vero cells were incubated in phenol red-freemaintenance MEM at 37°C in a 5% CO2 atmosphere in theabsence or presence of lactobacilli (1000 CFU/cell) for differenttime intervals: 4, 8, 12 and 16 hrs. Five hundred µl of culturesupernatants obtained after centrifugation for 10 min at 5000gwere mixed with an equal volume of PBS containing 1.12mMphenol red (Sigma) and 19 U horseradish peroxidase (Sigma).After 1h incubation at 37° C, the samples were brought to pH12.5 by the addition of 5µl NaOH 2 N and the absorbance wasread at 610 nm against a blank containing phenol red-freemaintenance MEM. Standard curves were made using H_2O_2 solutions in phenol red-free maintenance MEM to result in finalconcentrations of 0.5, 1, 2, 4, 8 and 16µM. The results, afterdeduction of control uninfected cell values, were expressed inµM H_2O_2.Lactic acid production by lactobacilli was evaluated with a commercial kit for the determination of D- and L-lactic acid (Test-Combination, UV-method; Boehringer Mannheim/RBiopharm, Darmstadt, Germany). The NADH increase, stoichiometric to the amount of D- and L-lactic acid, was determined by absorbance at 340 nm

RESULTS

Co-culture of lactobacilli and Vero cells

The interaction between lactobacilli and cultured eukaryotic cells was analyzed incubating sub confluent cell monolayers with bacteria in different growth phase conditions. Fig. 1shows the ability of microaerophilic strains of lactobacilli to survive and proliferate in the presence of Vero cells in aerobic atmosphere and antibiotic-free cell culture medium. After 24 h incubation all the Lactobacillus strains inoculated at 1000 CFU/cell, corresponding to the physiological concentration of lactobacilli in vaginal environment and to a late logarithmic growth phase, were viable maintaining the initial cell number of approximately 10 8 CFU/ml. Instead, lactobacilli inoculated in exponential growth conditions, corresponding to a 10 and 0.1 CFU/cell inoculum, increased their number by 2 to 4 logs. In the following48 hrs, the cell number of the faster growing strains (*L. salivariusand L. plantarum*) showed a significant decline. The pH value of cell culture medium after growth of lactobacilli was not modified in comparison to control at 24 and 48 hrs and only slightly lowered (from 0.3 to 0.4 units) after 72 hrs incubation.Microscopy inspection of cell monolayers did not show any change in cell morphology. Also Vero cell viability was unaffected by lactobacilli as verified by the uptake of the vital dye neutral red. Inhibition of HSV-2 infection by lactobacilli the antiviral potential of lactobacilli towards HSV-2 infection in cell cultures was studied measuring the virus yield after a single cycle of multiplication (Fig. 2). The effect ofbacteria on different steps of HSV-2 multiplication was analyzed by evaluating their ability to prevent virus infection when bound to the cell surface, to compete with virus adsorption to the cell membrane, and to reduce virus multiplication when present during virus replication. The activity was studied at different bacteria/cell ratio. Pretreatment of cell monolayers with lactobacilli for 1 h before virus adsorption resulted in very low inhibition of HSV-2 multiplication. A similar effect was observed after exposure of Vero cells to living lactobacilli for 24 hrs before infection (not shown). On the contrary, a straindependent inhibition of virus yield was observed when bacteria were present during virus adsorption (1h, 4°C), and removed before incubation at 37°C in antibiotic-containing medium to kill residual bacteria. The presence of *L. brevis*during virus binding to cell membrane receptors inhibited HSV-2 multiplication by more than 90%. Approximately 50% inhibition was exerted by *L. plantarum*, whereas low inhibition was shown by *L. salivarius*. To evaluate the effect of living lactobacilli on HSV-2 multiplication, bacteria were added immediately after the virus adsorption step and maintained during the virus replication cycle in antibiotic-free medium. Metabolically active lactobacilli showed good antiviral properties reducing virus yield by more than 90%. The three strains resulted equally effective towards HSV-2. The inhibitory effects were dependent on the bacteria/cell ratio. When a hundred-fold lower ratio (10 CFU/cell) was used only *L. brevis* produced a minor inhibition during HSV-2 adsorption (30% reduction) and multiplication (40% reduction). No inhibition was observed at 0.1 CFU/cell (data not shown).

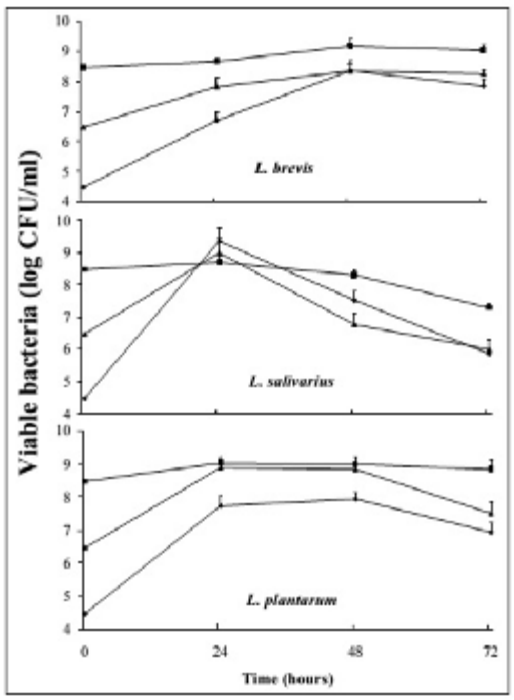

Fig. 1. Growth of lactobacilli on Vero cells. Bacteria wereinoculated on cell monolayers in antibiotic-free maintenanceMEM in 5% CO2 atmosphere. CFU were determined on MRSagar after different times of incubation. ■1000 CFU/cell; ▲10 CFU/cell; ◆0.1 CFU/cell.

Effect of lactobacilli and their culture supernatants (CSs) on virus infectivity

The inhibition exerted by lactobacilli during virus multiplication could be related to a direct effect on virus particles released from infected cells or on some intracellular event of virus replication. The effect on free virus particles was investigated titrating residual virus infectivity after incubation of high titre HSV-2 with bacterial cells or bacterial products. Virus titre was unaffected after preincubation of HSV-2 with each Lactobacillusstrain at a PFU/CFU ratio of 1/1000 (data not shown). To test the effect of overall Lactobacillusmetabolites on HSV-2 particles, antibiotic-free cell culture medium (MEM) from 16 h bacterial cultures in aerobic atmosphere were utilized. Culture supernatants obtained under these conditions (CSs) had a neutral pH and did not modify the infectivity of HSV-2 virions (data not shown).

Effect of CSs on HSV-2 multiplication Given that the anti-HSV-2 activity of lactobacilli was not exerted on virus particles, experiments were designed to verify whether the antiviral activity was exerted on intracellular events of virus multiplication. To this end, the overall effect of Lactobacillus products was evaluated using CSs (pH 7.2) as cell culture medium during HSV-2 multiplication. Virus yield was significantly reduced by all CSs although to a different extent. L. brevis-CS was the most effective causing more than 2 logs reduction (Fig. 3). To verify whether additional soluble antiviral factors could be also released by Vero cells upon incubation with Lactobacillus strains, parallel experiments were performed using culture supernatants of lactobacilli grown in the presence of cell monolayers (Cell-CSs). A similar level of HSV-2 inhibition was observed indicating that the antiviral activity is not mediated by a cell-released product.

Candidate antiviral components in CSs

The data previously reported seem to indicate that soluble factors released from lactobacilli inhibit intracellular HSV-2 multiplication. Hydrogen peroxide and lactic acid represent important Lactobacillus products with known antimicrobial activity towards several bacteria and mycetes. To verify the involvement of these metabolites in the above reported antiHSV-2 activity of lactobacilli, we first investigated the production of H2O2 and lactic acid in the experimental conditions used in the antiviral assay. Unlike L. brevis, bothstrains of *L. salivariusandL. plantarum*resulted good H2O2 producers in

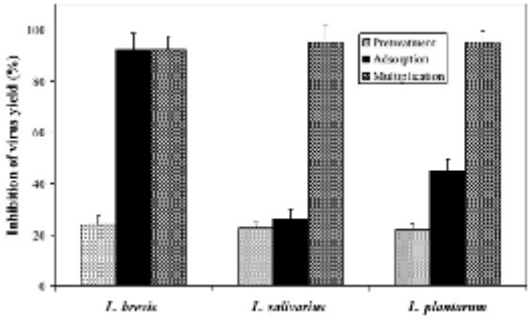

Fig. 2. Inhibition of HSV-2multiplication by lactobacilli. Verocells were incubated with acteria(1000 CFU/cell) for 1 h at 37°C inantibiotic-free maintenance MEMbefore virus infection (▨), duringHSV-2 adsorption for 1 h at 4°C (■), or for 16 hrs at 37°C aftervirus adsorption (▩). Antibioticswere added to pretreatment andadsorption samples during virusmultiplication. Results are expressedas % of PFU of control virus in thesame experimental conditions andeach value is the mean±SD obtainedfrom triplicate wells of twoindependent experiments. Controlvirus titre was ~ 6×10^6PFU/ml.

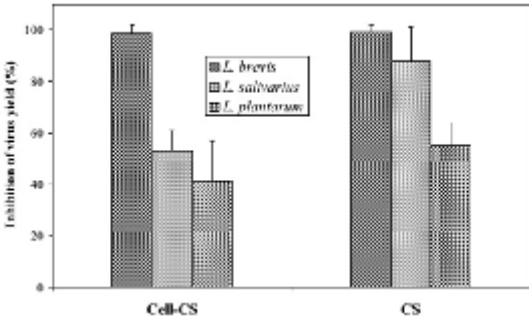

Fig. 3. Inhibition of HSV-2multiplication by culturesupernatants of lactobacilli.Supernatants of lactobacilli (4×10^8CFU/ml, 1000 CFU/cell) grown overnight in MEM, in the presence(Cell-CS) or absence (CS) of Verocells, were added to HSV-2 infected monolayers during the whole virus multiplication cycle. Virus yield wasdetermined by plaque assay. Resultsare expressed as % of PFU of controlvirus grown in the sameexperimental conditions and eachvalue is the mean±SD obtained fromtriplicate wells of two independentexperiments. Control virus titre was~ 2×10^6PFU/ml

bacterial growth medium (MRS) under anaerobic atmosphere[19]. In the antiviral test conditions, H2O2 was detected in µmolar concentration and the amount measured at 4 h intervals remained constant for each strain during the 16 h period (*L. salivarius* 2.2 µM; *L. plantarum* 0.7 µM). All the Lactobacillusstrains released substantial amounts of lactic acid (from 3.5 to 9.6 mM), although at different ratios of the two isomers. *L. brevisand L. plantarum*produced D- and L-lactic acid at approximately equimolar concentrations, whereas *L. salivarius* produced a fifteen-fold lower amount of the D isomer. The quantity of L-lactic acid measured in the presence of Vero cells and high producer Lactobacillus strains (*L.salivarius* and *L. plantarum*) was lower than expected considering the overall amounts produced by cells and lactobacilli. Indeed L-lactic acid concentration was lower thanthat produced by lactobacilli alone indicating a cell-mediated effect, probably due to cell metabolism. Altogether in cell culture medium and aerobic atmosphere lactic acid was produced in significantly lower concentration in comparison to that produced in the optimal atmosphere and growth medium(MRS) specific for lactobacilli. The antiviral potential of purified H2O2 and lactic acid towards HSV-2 was studied by evaluating both the effect on virions and the activity during virus multiplication in one cycle of virus growth (1 PFU/ cell, 16 h virus multiplication). Hydrogen peroxide is known to degrade in aqueous medium particularly in the presence of cultured cells[22]. Therefore, before studying the effect on virus multiplication, we determined the rate of H2O2 degradation in our

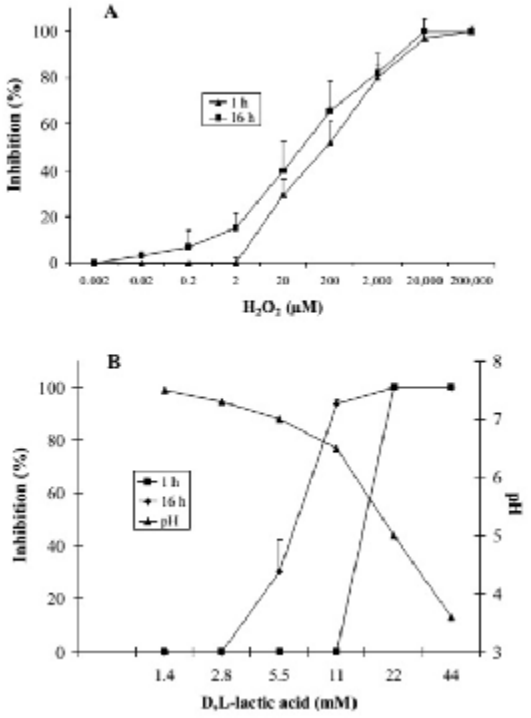

Fig. 4. Virucidal effect of H2O2 (A) and lacticacid (B). High titre HSV-2 (9×10^{6}PFU/ml) wasincubated in screw-cap tubes with differentconcentrations of compounds in maintenanceMEM for 1 h or 16 hrs at 37°C before titration byplaque assay. Results are expressed as % of PFUof untreated control in the same experimentalconditions and each value is the mean±SDobtained from triplicate wells of threeindependent experiments

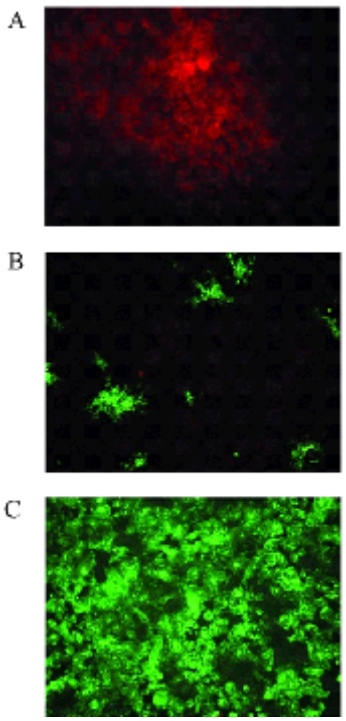

Fig. 5. Effect of lactic acid on HSV-2 antigen synthesis in Verocells. Lactic acid containing medium was added to infected cellsduring the whole virus replication cycle. A: uninfected; B: 22mM lactic acid; C: control virus. Viral antigens were labelledusing FITC-conjugated rabbit anti-HSV-2 immunoglobulins thatreact with the entire major viral envelope glycoproteins and at leastone core protein

experimental conditions. Micro molar amounts of H2O2 were reduced by 50% after 4 h incubation at 37°C with medium alone and to one tenth after 2hrs in the presence of Vero cells (data not shown). These resultsindicate that H2O2 is metabolized promptly in the cellenvironment and that it is not possible to maintain constantamounts of H2O2 on cell culture. Therefore only the virucidaleffect of H2O2 was studied. HSV-2 was incubated with H2O2orlactic acid in maintenance MEM to verify the role of bothmetabolites in the inhibition exerted by living Lactobacilluscells or CSs during virus multiplication. Hydrogen peroxideshowed a dose-dependent virucidal effect with a 50% inhibitionat 184 µM after 1 h incubation (Fig. 4A). Lactic acid showed atime- and dose-dependent virucidal effect that was directlyrelated to acidic pH (Fig. 4B). Exposure of herpes simplex virusto lactic acid amounts giving a pH 5.0 or lower for 1 hinactivated HSV and reduced virus titre by nearly 100% (3 and5 log inhibition for 22 and 44 mM, respectively). Viralinactivation was less effective at pH 6.5 (11 mM lactic acid),which resulted in 1 log inhibition of infection and requiredexposure to the acidic environment for 16 hrs. Exposure to5.5 mM lactic acid (pH 7.0) had little or no effect. Incubation of HSV in cell culture medium brought to identical acidic pH values with HCl, an unrelated mineral acid, produced the same levels of inhibition (data not shown). Moreover, when lactic acid solutions up to 44 mM were brought to pH 7.5 by NaOHaddition before incubation with virus no virucidal effect was observed. The inactivation of HSV by lactic acid was irreversible since infectivity was not restored by neutralization of the lactic acid-treated virus preparation (not shown). The antiviral activity of lactic acid towards intracellular events of virus multiplication was verified evaluating the viral antigen production by immunofluorescence (Fig. 5) in HSV-2-infected Vero cells incubated in lactic acid containing medium. HSV-2 antigen synthesis was reduced by more than 80% at 22 mM lactic acid (pH 5.8) and by approximately 40% at 11 mM(pH 6.9), while no inhibition was observed at 5.5 mM (pH 7.4). The pH values reported were those of infected cell culture medium after 16 hrs incubation in CO2 atmosphere. Neutralization oflactic acid containing medium suppressed the inhibitory activity.

DISCUSSION

Lactic acid-producing bacilli are part of the normal bacterial microbiota of the vagina and have a physiological role in maintaining a low pH (d”4.5) and protecting against invasion by other microorganisms. The mean vaginal lactic acid concentration of healthy women is 9.66 mM (from 4.7 to 17.7 mM) and lower lactate concentrations indicate severe depression of lactobacillary function[23]. Indeed, women with BV have a mean vaginal lactic acid amount of 2.8 mM [23] and a higher risk of acquiring HSV-2 infection[7]. The clinical observation of the inverse correlation between vaginal Lactobacillusload and genital herpes was not supported until now by in vitro studies confirming the role of lactic acid in theantiviral protection. The results presented here indicate that HSV-2 is irreversibly inactivated by concentrations of lactic acid giving pH values corresponding to that observed in the healthy human vagina. However, it must be noticed that in cell culture medium a pH value of ~ 4.5 is obtained using a lactic acid amount 3 fold higher than vaginal physiological concentration. This suggests that the buffering capacity of the vaginal milieu is different from that of culture medium or that the contribution of lactic acid to the vaginal acidic pH is only partial. The antiviral effect of lactic acid we observed is directly related to acidic pH values and to the time of exposure. Nicola et al.[24] demonstrated that HSV entry into Vero cells occurs via fusion at the plasma membrane and is inhibited by acid pretreatment ofvirions (pHs 4.7 to 6.0 in HEPES buffer), therefore it is possible that adequate concentrations of vaginal lactic acid are able to block the early stages of HSV-2 infection. Moreover, our study showed that intracellular events of virus multiplication are impaired in the presence of lactic acid amounts corresponding to the physiological concentrations of the compound in the vaginal environment. Vero cells exposed to lactic acid concentrations giving acidic pH bindi after HSV-2 resulted less effective in viral protein synthesis and in the production of infective virions. How this mechanism could act in the vagina is open to speculation. In fact, epithelial cells of the genital mucosal surface, that representthe initial target of HSV-2 infection, are covered by cervical mucus that provides a protective coating for the

vaginal and cervical epithelium[25]. However in healthy women the actual pH of the vaginal cell surface beneath the mucus layer, after removal of mucus with sterile cotton swabs, is 5.2-5.5 (C. Midulla, unpublished observations). Therefore it is possible that, in the presence of lactic acid producing vaginal microbiota, vaginal cells have a lower efficiency in supporting HSV-2 replication. It has been suggested that H2O2 produced by some Lactobacillusspecies may play some role, though not a crucialone, in controlling vaginal microbiota[26]. Although it is known that a H2O2 gas plasma sterilization process inactivates herpes simplex virus type1[27], no data are available on the virucidal effect of H2O2 on herpes simplex virus type 2. Our results demonstrated that H2O2 treatment impairs the infection capacity of HSV-2 virions. It is not possible to predict if such an activity could be effective in the vagina, since, to our knowledge, the vaginal concentration of H2O2 in women with H2O2-producinglactobacilli has never been determined. Moreover an effect of H2O2 on intracellular virus replication cannot be ruled out. Our results demonstrated that some Lactobacillus strains produce H2O2in cell culture giving micromolar amounts of the compound during the whole virus replication cycle. It is known that micromolar concentrations of H2O2 can produce different biological effects. Treatment with exogenous H2O2(1 µM) result in protein phosphorylation in U937-neo cells [28] and oxidative stress induced by 5 µM H2O2 in PC12 cells significantly upregulates neutral sphingomyelinase activity[29].

The results of the present study demonstrate that, beside the production of antiviral molecules, lactobacilli can protect susceptible cells from HSV-2 infection by other mechanisms. Vaginal lactobacilli were able to inhibit the first step of herpes virus infection. The antiviral activity exerted by the presence of lactobacilli during HSV-2 binding to the cell membrane was strain-dependent and appeared directly related to the adhesion capacity of Lactobacillusstrains [19]. In fact, *L. brevis*CD2, highly adhesive, was strongly inhibitory during HSV-2 binding, *L. plantarum* FV9 showed intermediate adhesiveness and inhibition, whereas L. salivariusFV2 adhering at low levels to the cell surface resulted a very poor inhibitor. Lactobacilli were unable to inhibit virus infection after preincubation with virions or cells before the adsorption step suggesting that bacterial strains did not bind to viral or cell surface molecules involved in the binding. Since the adsorption was performed at 4°C to allow binding but not entry of virus, we hypothesize that the simultaneous presence of virus and a great number of lactobacilli on the cell membrane impairs the fusion between viral envelope and the cell surface leading to an inhibition ofvirus entry into cells. Infection was significantly reduced if HSV-2 was cultured in the presence of living lactobacilli. The inhibition does not seem to be related to the presence of Lactobacilluscells since virusreplication is also inhibited if HSV-2 is cultured in cells fed with neutral pH culture supernatants of lactobacilli. It is unlikely that the inhibitory product in the CSs could be hydrogen peroxide or lactic acid since L. brevis CD2, the most active strain, does not produce H2O2 and neutralized lactic acid had no effect. The detected anti-HSV-2 activity of CSs, once the putative effects of lactic acid and H2O2 have been ruled out, raises the question of the chemical nature of active molecules. The identification ofthese compounds could give important contributes to the knowledge of natural defense mechanisms of the healthy human vagina against sexually transmitted viral infections. In conclusion, numerous mechanisms may be involved in the antiviral effect of lactobacilli towards HSV-2: interference withearly steps of virus infection (binding/entry), production of metabolites with a direct antiviral effect (lactic acid, hydrogen peroxide) and production of compounds able to inhibit intracellular events of virus replication.

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