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Antimicrobial activity of human cervical mucus

Waltraud Eggert-Kruse[1,4], Isabel Botz[1], Sabine Pohl[2], Gerhard Rohr[3] and Thomas Strowitzki[1]

[1]Department of Gynaecological Endocrinology and Reproductive Medicine, Women's Hospital, [2]Department of Microbiology and Hygiene and [3]Department of Internal Medicine IV, Klinikum Mannheim, University of Heidelberg, Heidelberg, Germany

[4]To whom correspondence should be addressed

The antibacterial activity of human cervical mucus (CM) was examined on standardized microbial colonized agar plates (agar diffusion test). In parallel, the lysozyme content of CM was determined by means of a turbidimetric test system in aliquots of the same CM specimens. Suspensions of living lyophilized *Micrococcus lysedeikticus* were used as bacterial substrate. Testing was performed in a total of 133 CM samples, obtained at mid-cycle from sexually active women from unselected infertile couples with a median age of 30 (range 21–42) years. All mucus specimens showed considerable antibacterial activity with clearly visible circular inhibition zones around the CM-filled holes in the colonized agar plates. Related to the effect of hen's egg white (HEW)-lysozyme on the same plates, the median activity of the CM specimens in the agar diffusion test was equivalent to 33.0 (range 6.4–391.4) μg/ml HEW-lysozyme. However, there was a wide inter-individual range of antibacterial effects of cervical secretions. The cervical index did not significantly influence the outcome of either test. The pH of the endocervical CM also was not correlated with the antibacterial effect. Sexual activity leading to the presence of spermatozoa in CM considerably increased its antibacterial effect. The activity was markedly higher in samples obtained within hours after intercourse compared with those taken after sexual abstinence of ≥5 days ($P < 0.05$). In microbially colonized CM specimens compared to sterile CM, all obtained under hormonally standardized conditions, the antibacterial activity in the agar plate test was significantly lower ($P < 0.05$). The results of this pilot study demonstrate the considerable antibacterial activity of human CM.

Key words: antimicrobial activity/cervical factor/cervical mucus/lysozyme/sperm–mucus interaction

Introduction

The cervix regulates sperm transport to the site of fertilization and protects the upper female genital tract against invading micro-organisms. Whereas cervix and vagina are usually polymicrobially colonized and are known to be ecological niches with a specific transient and resident flora, the uterine cavity is usually sterile (Levinson *et al.*, 1977; Sparks *et al.*, 1977). Cervical mucus (CM) is known as a multifactorially determined filtering system but not much is known about its antimicrobial activity. This might partly be due to rheological characteristics and difficulties in handling this viscous hydrogel. Furthermore, CM is available only in small amounts and is influenced by several variables, such as endocrine status regulating the characteristic cyclic changes and pH.

The natural defence system of the uterine cervix is important because ascending infections, e.g. with micro-organisms transmitted via sexual intercourse (sexually transmitted diseases) are a major cause of female morbidity, adverse pregnancy outcome and infertility (Weström, 1994; Paavonen and Eggert-Kruse, 1999; Ralph *et al.*, 1999). In intrauterine insemination (IUI) and other methods of assisted reproductive technology, the potential barrier effect of the uterine cervix is circumvented. It is unclear if inherent defects of the cervical barrier system are responsible for endometrial colonization and inflammation in some women (Egbase *et al.*, 1999; Viniker, 1999).

In the present investigation, the antibacterial activity of human CM, with special attention to lysozyme as an important enzymatic factor, was determined by bioassay in a large series of freshly obtained CM samples. Furthermore, potential causes of CM inter-individual variation were analysed in this pilot study.

Materials and methods

Cervical mucus (CM) samples

The antibacterial activity was determined in CM samples obtained at midcycle from 122 women with a median age of 30 (range 21–42) years (total samples $n = 133$). All women, as well as their male partners, were without symptoms of genital tract infection at the time of examination. None of them had pathological vaginal or cervical discharge, and cervices had a macroscopically normal appearance. Women presented at the outpatient clinic because of the couples' infertility of at least 1 year's duration, and none of the women used any method of contraception.

Mucus samples were taken in the peri-ovulatory period (median day 14), determined with basal body temperature (BBT) or ultrasound, of spontaneous cycles, after hormonal stimulation for ovulation induction, or after oestrogen treatment to standardize CM quality before diagnostic tests, e.g. in-vitro sperm–CM penetration testing (SCMPT), as described previously (Eggert-Kruse *et al.*, 1989a,b). The cervix was exposed with a sterile speculum and vagina and ectocervix were first cleaned with large, dry and sterile swabs before endocervical mucus was carefully obtained with a special device (Aspiglaire®, IMV, L'Aigle, France).

The cervical index was determined (Insler *et al.*, 1972), including amount of CM, condition of the cervical os, spinnbarkeit and ferning. The pH of the CM was examined with paper strips using two overlapping ranges (5.4–7.0 and 6.4–8.0) after saturation of the material (pH Indikator Papier®, Merck, Darmstadt, Germany). This method was validated by comparing the colorimetric procedure with electrometric measurements by means of a glass-electrode which provided correlation coefficients (r) of >0.9 ($P < 0.001$) (Eggert-Kruse *et al.*, 1993a).

Nearly half of the mucus samples were obtained shortly after sexual intercourse (8–12 h later), the other specimens after a longer period of sexual abstinence (at least 5 days), or at SCMPT or at a routine gynaecological examination. All samples were checked for the presence of spermatozoa using the high power field (HPF) (×400) of a standard light microscope. In samples obtained after intercourse, the number of motile spermatozoa in CM was counted (mean of 20 visual fields).

To screen CM samples for microbial colonization, a sterile cotton swab was introduced and inoculated into a standard transport medium (Port-a-Cul Universal®, Becton Dickinson, Heidelberg, Germany). Microbial cultures and bacterial identification were performed according to standard criteria (Department of Microbiology and Hygiene, University of Heidelberg, Germany) using inoculation of blood agar, MacConkey agar, thioglycolate bouillon, Schaedler plates, kanamycin-vancomycin plates, and Sabouraud plates for culture of yeasts. Additionally, calibrated sterile plastic loops were used to inoculate the material in Shepard's U 9 medium for culture of mycoplasmas. Endocervical material was screened for *Chlamydia trachomatis* (McCoy cell culture), and vaginal fluid was examined for *Trichomonas vaginalis* (phase-contrast microscopy).

If necessary, CM samples were stored in the refrigerator at 4°C (maximum of 1 week) before further processing to examine the antibacterial effect. A subgroup of specimens was additionally stored for a longer period (up to 38 days) at 4°C and at –20°C, to evaluate a possible influence of storage conditions.

Evaluation of antimicrobial activity

To determine the antibacterial effect of cervical secretions, CM samples were examined in a modified agar diffusion test. The procedure was comparable with testing for antimicrobial activity after specific chemotherapy. As bacterial substrate, micrococci (*Micrococcus lysedeikticus*) were used because of particular sensitivity against lysozyme (Fleming, 1922).

Briefly, agar plates with defined volume (25×25 cm by 2 mm) were carefully prepared in a special apparatus (metal frame with two plain glass plates). Antibiotic medium 3 (Grove and Randall standard medium; Merck, Darmstadt, Germany), which was supplied with 1% agar–agar powder (Merck) (pH 7.0), diluted in distilled water, was used according to standard procedures for medium preparation. Before plating, the agar solution was inoculated with 40 mg of lyophylized *M. lysodeicticus* ATCC 4698 (Sigma Chemicals, Deisenhofen, Germany). This was resuspended in 3.5 ml CASO bouillon (peptin from casein and from soya, glucose, sodium chloride, dipotassium hydrogen phosphate; Merck) and vortexed; 3 ml of this suspension was added to the antibiotic agar, and 0.5 ml was used to define the number of colony forming units (CFU) in the *M. lysodeikticus* suspension.

A dilution series was prepared with physiological saline, spiralized (Spiral system®, Meintrup Laboratory Supply, Lähden, Germany) on CASO agar (Merck), and examined for bacterial growth after incubation. The mean of duplicate readings on each plate gave an overall median concentration of 6×10^{15}/CFU/ml.

For liquefaction of the mucus, sonification (Branson Sonifier B-15, Heusenstamm, Germany), after addition of phosphate buffered saline (PBS), pH 7.2 in 1:1 dilution, for 3×10 s with 20 s intervals, was used (Eggert-Kruse *et al.*, 1993b). Circular holes of defined volume (radius 3 mm) were cut in the agar plates with a special device, and were filled with 50 µl of liquefied CM. The size of the plate allowed preparation of 8×8 = 64 holes. Sterile conditions for preparation of the test system were always ensured.

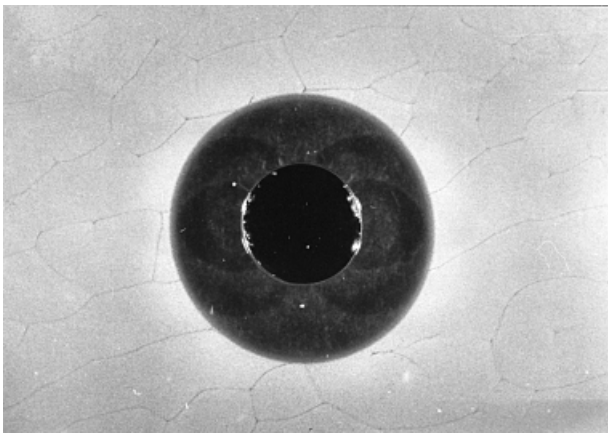

Figure 1. Clearly visible typical inhibition area around a hole in the bacterially colonized agar plate, which was filled with human cervical mucus obtained after oestrogen treatment (incubation time 22 h).

For each CM sample, at least two holes were filled (in most cases in different agar plates) to allow multiple testing. Negative controls (distilled water, PBS pH 7.2) and positive controls [hen's egg white (HEW)-lysozyme (EC 3.2.1.1 7, Boehringer, Mannheim, Germany) in PBS solution, in eight different concentrations covering a range up to 1000 µg/ml] were also included on each plate.

After incubation for 22 h at 30°C, homogeneous bacterial growth (white opaque surface) and clear inhibition areas (transparent zone) around the CM-filled holes (Figure 1) and the HEW-lysozyme standards were clearly visible. The diameter of the inhibition zones (IZ) was measured (Mebprojektor, Behring, Marburg) (precision of 0.1 mm reading), the mean of (at least) duplicate readings of the CM aliquots was taken for analysis. All readings were done by one observer (I.B.) throughout the study.

For better comparison, the antibacterial action of the CM specimens, as determined by impairment of bacterial growth on the agar plates, was related to the antimicrobial effect (inhibition zone) caused by HEW-lysozyme solution of defined concentration by means of a standard curve (expressed as HEW-lysozyme equivalent activity).

Determination of the lysozyme concentration in a photometric assay

In parallel to the agar diffusion test, the lysozyme-related antibacterial effect of CM samples was determined by means of a commercial turbidimetric test system (Testomar Lysozym Mono®, Behringwerke, Marburg, Germany). This assay is based on a previous method (Prockop and Davinson, 1965). Briefly, it uses the ability of lysozyme to interact with the outer bacterial cell membrane of *M. lysodeikticus* in standardized suspensions resulting in a reduction of the turbidity which is proportional to the light absorption ($\delta E = E1 - E2$), before and after addition of the test material. This was measured photometrically at 546 nm (Zeiss, Oberkochen, Germany). The assay was performed according to the manufacturer's instructions. The suspension of living lyophilized *M. lysodeikticus* used was equivalent to 50 000 international units (IU)/mg according to Shugar (Shugar, 1952) in a PBS solution of pH 7.2 (product information).

To ensure the pipetting of exact volumes, liquefaction of the CM samples was performed with high frequency ultrasound (sonifier) as

described above. The absorbance of the lysozyme reagent was read after 30 s and again after 2 min after addition of the material. The lysozyme equivalent activities were calculated by means of the absorbance differences obtained from a standard curve in μg/ml of HEW-lysozyme. Standards were: negative controls, bidistilled water and PBS pH 7.2; positive controls, egg white lysozyme of defined concentration (provided in the kit). These were included in all of the test series. As an additional precision control for the test system, lyophilized human serum with defined biochemical properties was used (Kontrollogen L®, Behringwerke). The mean of duplicate measurements, obtained by the same observer (I.B.) throughout the study, were taken for further analyses.

Measures of variability

In samples tested on several occasions (after 1, 2, 3 and 4 weeks), no marked influence of storage conditions at +4°C (and additionally at –20°C) was found on antibacterial activity in the agar diffusion test. For example, a correlation coefficient (r) of 0.954 ($P < 0.001$) was obtained for samples tested for a storage time of 22 days (4°C); r was 0.907 ($P < 0.001$) with regard to the turbidimetric assay ($n = 18$).

The correlation coefficient for results obtained after separate liquefaction procedures (sonification) of CM aliquots from the same samples, tested on different agar plates, was 0.976 ($P < 0.005$) ($n = 20$). Duplicate determination of lysozyme activity in CM aliquots (after separate preparation) in the photometric test also showed high correlation, with $r = 0.971$ ($P < 0.005$). In both assays, the intra-assay variation was <7%, with an inter-assay variation of <14% for CM, and <6% for HEW-lysozyme/PBS solution.

The results obtained using the control human serum with defined enzymatic concentration in the turbidimetric test were within the confidence range, with an inter-assay variation of <7% ($n = 9$).

Statistical analysis

For statistical analysis, Spearman rank correlation, Wilcoxon rank sum and Kruskal–Wallis tests, and for dependent variables Friedman–Wilcoxon tests were used. The level of significance was set at $P < 0.05$.

Results

Antimicrobial activity of cervical secretions

Outcome of the agar diffusion test

The results demonstrate the antibacterial activity of cervical secretions in biological test systems. With regard to the agar diffusion test, all 133 CM samples inhibited the growth of *M. lysodeikticus*. After an incubation period of 22 h, the circular inhibition zones around the holes in the agar plates filled with solubilized CM were clearly visible (see Figure 1). No antimicrobial effect (i.e. no inhibition zone) was seen when holes were filled with PBS or distilled water (negative controls).

HEW-lysozyme (positive controls) with a concentration/activity of 30 μg/ml gave an inhibition zone with a median diameter of 19.1 mm ($n = 9$). When the inhibition zone caused by endocervical mucus was related to the activity of lysozyme/PBS solution on the same 8×8 hole agar plate using a standard curve, the median antibacterial activity of solubilized CM was equivalent to 33 μg/ml HEW-lysozyme ($n = 133$). The activity was highly variable with a range of 6.4–391.4 μg/ml.

Results of the turbidimetric test and correlation of both assays

All samples were examined in parallel in a turbidimetric test system. In this assay, after a contact time of 2 min, all but two samples exhibited detectable activity resulting in measurable reduction of turbidity of the bacterial suspension. Again, the degree of bacteriolytic effects was variable within a wide range of 0–253 μg/ml, with a median value equivalent to 9.8 μg/ml related to HEW-lysozyme in this assay ($n = 133$). No bacteriolytic activity (lysozyme concentration equivalent 0 μg/ml) was seen when PBS and distilled water were used.

Although the lysozyme-related activity values obtained in the turbidimetric assay after 2 min were significantly lower than the antimicrobial activity determined in the agar diffusion test ($P < 0.001$) (Wilcoxon test for dependent variables), the outcome of both test systems was significantly correlated ($r = 0.844$; $P < 0.001$) (Spearman rank correlation).

Consideration of influencing factors

Influence of sexual intercourse

Sexual intercourse and the presence of spermatozoa in CM considerably influenced the detectable antibacterial effect of cervical secretions in both test systems. Overall, 53/133 (39.8%) of the samples were obtained shortly after intercourse (maximum 12 h) and contained motile spermatozoa. These samples were compared with CM specimens taken after a period of sexual abstinence of at least 5 days. Mucus samples taken after intercourse, but without spermatozoa were excluded from these analyses. The median antibacterial activity was significantly higher in the postcoital CM samples: agar plate testing showed an antibacterial effect equivalent to a median concentration of 39.4 (range 6.4–354.6) μg/ml HEW-lysozyme ($n = 53$), compared with 27.4 (11.4–326.0) μg/ml, in specimens obtained after sexual abstinence ($n = 60$) ($P < 0.05$). The median number of motile spermatozoa was four per HPF. The antibacterial activity did not increase with the number of visible spermatozoa and in fact appeared higher in samples with few (47.2%, $n = 25$) compared with CM specimens containing many spermatozoa (52.8%, $n = 28$) with median values of 49.2 and 34.5 μg/ml HEW-lysozyme equivalent respectively ($P < 0.01$).

The influence of sexual activity was confirmed when only CM samples obtained after standardized oral oestrogen treatment were considered. The bacteriostatic effect was again higher in CM specimens with spermatozoa (median activity related to HEW-lysozyme 35.7 μg/ml) ($n = 29$) compared to those samples obtained after sexual abstinence ($n = 40$) with a median value of 25.7 μg/ml ($P < 0.05$).

Eight of the women were compared for the antibacterial activity of their cervical secretions on two occasions in one cycle under controlled hormonal conditions: (i) after sexual abstinence and (ii) within 10 h after intercourse. After 7 days oestradiol administration and after a period of sexual abstinence of at least 5 days, the antibacterial effect of CM related to HEW-lysozyme was equivalent to a median of 29.8 μg/ml, and in the post-coital samples, taken 3 days later, after continuous oestradiol treatment, it appeared higher with a median value of 37.8 μg/ml, although the sample was too small to be statistically analysed.

The influence of coitus was also obvious when CM was examined in the turbidimetric assay. Median values of

bacteriolytic effect related to HEW-lysozyme were nearly twofold higher in postcoital samples (median 11.4 μg/ml) than in CM specimens obtained after sexual abstinence (median 6.0 μg/ml) ($P < 0.05$). Again, the antibacterial action did not increase with the number of spermatozoa, with higher activity values in CM containing few spermatozoa on microscopical examination (15.0 μg/ml HEW-lysozyme equivalent) than in samples with many spermatozoa (median 6.4 μg/ml respectively) ($P < 0.01$). When only results of women who received oral ethinyloestradiol (to standardize the CM quality) were considered in the photometric test system, the median lysozyme equivalent effect was again significantly higher in postcoital samples (median 9.8 μg/ml), compared with specimens from the abstinence group (median 5.5 μg/ml HEW-lysozyme equivalent) ($P < 0.05$).

In CM of the eight women who were studied on two occasions in one cycle after oestradiol treatment, the antibacterial activity was equivalent to a lysozyme concentration in this test of 7.4 and 10.8 μg/ml after sexual abstinence respectively.

Influence of the CM status (cervical index)

No significant correlation of the antibacterial effect of CM and patients' cervical index (CI) was observed. The condition of the CM (defined by Insler *et al.*, 1972) showed a median score of 12 (maximum index value) and a minimum of 8. As expected, the cervical index was higher in patients after oestrogenic stimulation (median 12), and in CM obtained in spontaneous cycles (median 11.5) than in patients using medication to improve follicular growth, mostly anti-oestrogenic substances, e.g. clomid, with a median score of 10 (not significant).

After exclusion of samples obtained after intercourse, the antibacterial activity of CM in the agar diffusion test appeared slightly higher in the group with reduced CI (≤10) (13.3% of samples), compared with the other samples having a good CI (>11) [median 33.9 (11.4–228.4) and 25.7 (12.4–326.0) μg/ml HEW-lysozyme equivalent respectively], without achieving statistical significance. In the photometric test as well, there was no significant difference in the lysozyme concentration-related effect when samples with reduced CM condition were compared with samples of excellent quality.

Influence of cervical mucus pH

The antibacterial activity of CM was not significantly related to the pH of cervical secretions. Immediately after CM samples had been obtained from the endocervix, the pH could be determined in a total of 95 samples (71.4%). The median pH was 7.0, with a range of 5.4–8.0. Confirming previous findings (e.g. Eggert-Kruse *et al.* 1993a), there was a significant influence of the CM pH on the number of motile spermatozoa in postcoital mucus ($P < 0.05$); therefore only specimens of the abstinence group were considered for analysis [median pH in this subgroup also 7.0 (range 5.4–7.5)]. Comparison of three groups: CM specimens with a reduced pH (<7) (25%), pH = 7 (40.9%) or > 7 (34.1%), did not reveal significant differences of antibacterial activity in the agar diffusion test as well as in the turbidimetric assay.

Influence of hormonal medication

In the population of women who received oral oestrogen medication (oestradiol 80 μg/day) to standardize the endocrine influence on CM quality, two groups were compared: patients with ≤8 days of oral oestrogens ($n = 38$) (46.3%) and women with a higher total oestrogen dosage (≥9 days, median 11 days treatment) ($n = 44$) (53.7%). The antibacterial activity in the agar plate test was markedly higher in samples obtained after longer hormonal treatment [median HEW-lysozyme equivalent effect 35.6 (range 14.0–184.8) and 26.8 (13.4–163.4) μg/ml for higher and lower oestradiol doses respectively], although this did not achieve significance.

In the turbidimetric assay also, the antibacterial effect of CM was considerably higher in the group of specimens obtained after longer rather than shorter oral oestrogen treatment (median lysozyme concentrations 11.6 and 6.0 μg/ml respectively; $P < 0.01$, Wilcoxon test).

In both test systems, no significant difference was found for the antibacterial effect of CM samples obtained in the periovulatory period of spontaneous cycles when compared to CM specimens of women with oral oestrogen treatment, although it was higher in the oestradiol group. The median HEW-lysozyme equivalent antibacterial activity was considerably increased in the small group of CM samples of patients receiving other hormonal medication [median 47.6 (range 15.6–326.0) μg/ml, $n = 10$]; this was mostly related to clomid ($P < 0.05$).

Similar differences were seen when women with a reduced CM quality (CI ≤10) were further excluded from analysis, without significant differences of antibacterial activity in CM samples of women receiving oestradiol compared to CM samples obtained in spontaneous cycles (with lower values in the untreated group), but higher median activity in the small group of women with anti-oestrogenic medication ($P < 0.05$).

Relationship with microbial colonization

Microbial cultures showed aerobic bacterial growth in 63% of samples, including commensal aerobes (mostly *Staphyloccocus epidermidis*, or apathogenic streptococci), and potentially pathogenic species (mostly *Escherichia coli*, group B-streptococci, *Proteus* spp., *Klebsiella* spp., and many other species with a lower prevalence). Additionally, lactobacilli were cultured in the majority of samples. Yeasts were found in 8.9% of women in the fornix posterior vaginalis. The prevalence of mycoplasmas was low (<5%). As samples were not kept under strictly anaerobic conditions, there was also a low rate of potentially pathogenic anaerobes (<5%) (not considered for analysis). Screening for *Chlamydia trachomatis* in endocervical material and for *Trichomonas vaginalis* in vaginal fluid was negative in all women.

To evaluate a potential relationship with microbial colonization of CM, only samples obtained after sexual abstinence were considered. In the agar plate test, the median activity against the indicator strain was significantly higher in sterile CM samples compared with CM specimens containing aerobic bacterial growth [31.2 (range 15.6–326.0), equivalent to HEW-lysozyme, and 24.1 (range 11.4–233.2) μg/ml respectively; $P < 0.05$]. This was mostly related to potentially pathogenic species (37.3% of samples).

This tendency was confirmed when only samples obtained after oestradiol treatment to standardize the endocrine influence on the mucus properties were considered. Sterile samples had a significantly higher antibacterial activity than samples colonized with aerobic micro-organisms in this subgroup (effect equivalent to a median HEW-lysozyme concentration 28.2 versus 24.0 µg/ml respectively; $P < 0.05$ Wilcoxon test).

In the turbidimetric assay as well, the bacteriolytic effect was stronger (nearly 2-fold) in sterile CM specimens compared with mucus samples with aerobic growth in microbial cultures, but differences in the outcome of this test did not achieve statistical significance.

Discussion

The uterine cervix is considered to be a barrier system against bacteria ascending the upper female genital tract due to its specific anatomical structure and the production of CM. Cervical mucus is a multifactorially determined microfilter system with characteristic cyclic changes. Apart from immunoglobulins (Ig) and proteins originating from serum transudation and the local immune system, particularly the secretory IgA, enzymatic factors play a major role. However, there are only few studies related to the enzymatic properties of human CM. Therefore, a large series of freshly obtained CM samples was examined for its antimicrobial activity in the present investigation using two different biological test systems.

Lysozyme is an important factor for the antibacterial action of many body fluids, with high concentrations in, e.g. tears, saliva, serum, colostrum, amniotic fluid and vaginal secretions (Pommerenke and Taylor, 1953; Davis *et al.*, 1968; Bergmann *et al.*, 1972; Hyslop *et al.*, 1974; Tustanowski *et al.*, 1978). In synergism with local Ig and factors of the complement cascade (Schumacher, 1974) this enzyme causes immune bacteriolysis, and is part of the non-specific defence system against micro-organisms (Glyn and Milne, 1967; Schumacher, 1974; Rebello *et al.*, 1975; Schumacher *et al.*, 1977; Chantler and Elstein, 1986; Haas, 1986). Human lysozyme is a basic protein with a polypeptide chain of 130 amino acids structured by four disulphate bonds with a molecular weight of ~15 000, and acts by hydrolysis of glycosidic bonds, e.g. as muramidase (Salton, 1957; Imoto *et al.*, 1972; Phillips, 1974; Weisner, 1983). However, enzymes other than lysozyme are also involved in the antimicrobial action of genital secretions, e.g. lactoferrin (Alsen and Leiberman, 1972).

The results of this pilot study demonstrate that human CM has a considerable antibacterial effect. A large series of 133 mucus samples was evaluated, and all specimens showed clearly measurable inhibition of bacterial growth on the agar plates. For means of comparison, the resulting inhibition zones were related to the effect of HEW-lysozyme/PBS solution of defined concentration on the same agar plates.

The antibacterial effect of human CM was evaluated in early studies (e.g. Pommerenke and Rochester, 1946; Pommerenke and Taylor, 1953) and later (Rozanski *et al.*, 1962; Enhörning, 1970; Zukerman, 1975), without direct relationship to lysozyme. However, in these investigations, only a small number of samples was tested, and not much attention was paid to general CM quality and potentially influential factors, such as endocrine effects, the pH of CM, micro-organisms colonizing the cervical os, and sexual activity before CM samples were taken. Exogenous variables with probable influence on enzymatic properties were also poorly considered: e.g. storage conditions, and particularly the liquefaction procedure. These early studies used unsolubilized CM or CM after treatment with physiological saline, which has a high variability and does not allow pipetting in exact volumes. Although in this study repeated measurements of the same samples after different periods of storage at 4°C and after freezing did not reveal a marked impact on the antibacterial activity, no frozen–thawed, only fresh samples were used in the present investigation.

As CM is a hydrogel, solubilization is necessary to ensure a homogeneous mixture and standardized rheological properties, which are important, e.g. for the diffusion rate (Peters and van Trappen, 1977) in the agar plate system. Sonification is an easy method for liquefaction and dispersion of CM, and proved to be practical in this as well as in previous studies (van Kooij, 1984; Eggert-Kruse *et al.*, 1993b). It was used in all samples. To exclude changes in the macromolecular structure induced by sonification with subsequent loss of activity, a series of lysozyme standards were treated in the same way which did not result (in both assays) in reduction of the antibacterial effect compared to lysozyme solution aliquots without sonicator treatment. The 'classical' method for liquefaction of mucus is the enzymatic treatment with bromelin (Ingerslev and Poulsen, 1980). This cannot be recommended before evaluation of antimicrobial effects of CM in biological tests, because of proteolytic activity (van Kooij, 1984; Rowan *et al.*, 1990) and its significant interference with the biological tests used in this study (data not shown).

The different approaches to evaluate the antibacterial effects of human CM in the present study markedly influenced the results. The outcome of agar plate testing, adjusted to the growth kinetics of *M. lysodeikticus* as indicator strain, e.g. with regard to the number of inoculated bacteria, nutritional contents as well as temperature and incubation time differed in many respects from the commercial turbidimetric assay with a contact time of 2 min. Not only have the completely different kinetics of both systems to be considered, but also the stronger antibacterial effect in the agar diffusion test may be related to non-specific effects of basic macromolecules in CM on the agar plates, diffusion variables, or the presence of other antimicrobially acting substances, which might interfere with the growth kinetics of *M. lysodeikticus*, but not necessarily lead to lysis of bacterial cell membranes. Synergistic action of lysozyme with Ig and complementary factors may be effective only in the immunodiffusion test; furthermore, interference of CM with the nutritional content of the medium is possible. The much longer contact time (22 h) in the agar diffusion test compared to 2 min in the photometric assay may also play a considerable role. In addition, it should be noted that the effect against micrococci in both test systems was related to the action of egg-white lysozyme, which was always evaluated in parallel. There are interspecies differences for this enzyme, and the activity of human lysozyme is estimated

to be much stronger (at least 2-fold) compared to HEW-lysozyme (Osserman and Lawlor, 1966; Weisner, 1983). Although the outcomes of both procedures were significantly correlated ($P < 0.001$), results were considered separately for analysis of endogeneous factors with potential influence on the antimicrobial CM effects.

The endocrine influence is of particular importance to the condition of CM. The majority of samples were obtained under hormonally standardized conditions at mid-cycle, after oral oestrogen treatment. This ensures a high quality CM with usually good penetrability, which is independent of the endogenous endocrine status (e.g. the hormonal pattern in the early follicular phase) (Eggert-Kruse *et al.*, 1999). Oestrogen induced changes of microcapillary permeability resulting in increase of water in CM (Wolf *et al.*, 1978), with consequently lower enzymatic concentrations expected. A decrease in Ig at mid-cycle has been reported (Moghissi and Neuhaus, 1962; Schumacher *et al.*, 1965; Chantler and Elstein, 1986). However, this was not observed in the present study. The antibacterial activity was stronger after oral oestradiol administration than in spontaneous cycles and increased in women with longer oestrogen treatment, which is consistent with higher levels of lysozyme during the oestrogenic phase of oral contraceptives (Schumacher, 1974; Schumacher *et al.*, 1977). In animal studies, the highest concentration of lysozyme in secretory granula of endocervical cells was found under oestrogen dominance, with marked reduction after ovariectomy (Nicosia *et al.*, 1984). It might be postulated that oestrogens stimulate the local production of antimicrobial factors to compensate for reduced mechanical filtering capacity of the mucous network in the peri-ovulatory period. Future research along this line is necessary to evaluate this clinically important effect.

The status of CM is a dynamic parameter. Classically, the quality of CM is determined using a cervical scoring system, e.g. the cervical index (Insler *et al.*, 1972). The cervical index did not significantly influence the antibacterial effect of the CM, but the small number of specimens with an Insler score <10 has to be taken into account. Due to concentration effects, higher activity may be expected in viscous samples with low volume. In the present study no 'cervical factor' patients were included. Antisperm antibodies in cervical secretions cannot be excluded, but the prevalence in unselected populations is low (Eggert-Kruse *et al.*, 1993b) and there is no convincing rationale for interference with antimicrobial effects. The pH of mucus is also an important factor which might influence the diffusion rate in the agar plate test as well as the activity of lysozyme (Saint-Blanchard *et al.*, 1970; Peters and van Trappen, 1977) (alkaline pH leading to increase of lysis rate). The median pH in CM samples examined here was consistent with findings in much larger populations (Eggert-Kruse *et al.*, 1992). The pH of CM may impair sperm–mucus interaction, and is related to the endocrine situation (Jenkins *et al.*, 1989; Eggert-Kruse *et al.*, 1993a). However, it did not significantly interfere with the antimicrobial activity in both assays.

Results are restricted to women of unselected infertile partnerships, and potential differences in relation to a population of 'normal' fertile individuals cannot be excluded. It is difficult to examine a large group of sexually active women of that age group not using a contraceptive, which is bound to influence the CM condition by, e.g. chemical agents, progesterone activity of oral contraceptives, the copper content or other changes caused by intrauterine devices (Jonsson *et al.*, 1991). However, it is interesting to note that CM samples of patients examined here who could be followed for subsequent fertility ($n = 93$), did not show significantly different antibacterial effects depending on whether they were from women with or without a subsequent pregnancy within 1 year (controlled for other infertility factors).

A strong influence of sexual activity was seen with significantly stronger antibacterial effect in the postcoital CM samples. Cervical index and pH, as well as microbial prevalence did not differ between samples with and without spermatozoa. Differences might be caused by unspecific sexual stimulation, or semen–mucus contact, reflecting lysozyme from seminal plasma. Semen is known to contain lysozyme and other factors as part of the non-specific local defence system (Taylor and Morgan, 1952; Razin and Rozansky, 1957; Schumacher, 1974). Involvement of this enzyme in the oocyte penetration ability of spermatozoa has also been suggested (Zanefeld *et al.*, 1972). Furthermore, the increase of antibacterial effects might be related to non-specific activation of lysozyme in CM by basic seminal proteins, or release of lysozyme and/or other antimicrobial factors from white blood cells present in semen, or originating from the postcoital increase of white blood cells in the female genital tract (Pandya and Cohen, 1985; Parkhurst and Saltzman, 1994).

Consistent with previous studies, micro-organisms were cultured in a considerable proportion of CM samples although all women were without visible inflammatory changes of the cervix or vagina and without other signs or symptoms of upper genital tract infection (Eggert-Kruse *et al.*, 1992). A significant association of antimicrobial activity and aerobic colonization of CM was noted in this investigation. There was also a stronger effect in samples colonized with potentially pathogenic bacteria than with species of the physiological flora. However, the evaluation of antibacterial effects in this study was limited to the impact on *M. lysodeikticus*, which is the optimal substrate to study lysozyme effects. The action of lysozyme on Gram-positive bacteria is stronger than on Gram-negative species, the effect on yeast and on viruses is less clear (Tobgi *et al.*, 1988; Cisani *et al.*, 1989). The potential relationship with anaerobic growth is uncertain because the routine procedure for microbial cultures was used which is unfavourable for strict anaerobes. It is well known that anaerobic growth in genital secretions is highly dependent on transport and culture conditions, with, e.g. prevalences of potentially pathogenic anaerobes in $>70\%$ of semen samples, when special attention is paid to these bacteria (Eggert-Kruse *et al.*, 1995).

Although some antibacterial effects of human endocervical mucus were demonstrated in the present investigation, future studies are necessary to examine the action of CM against other micro-organisms, to evaluate further antimicrobial factors in cervical secretions other than lysozyme, and to analyse further determinants of intra- and inter-individual variability which might potentially lead to therapeutic consequences.

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