The majority of pregnancies continue until 37 to 42 weeks of gestation despite potentially harmful bacteria that are present nearby in the vagina. This suggests that competent local defense mechanisms protect the feto-maternal unit from ascending vaginal bacteria. Such mechanisms may include antibacterial properties of the amniotic fluid, inhibitory effects of the fetal membranes, antibacterial properties of the cervical mucus, and mucosal immune factors. Microbial invasion of the amniotic cavity can be found in approximately 30% of women with preterm prelabor rupture of the membranes, and up to 20% of women with premature labor and intact membranes have bacterial infection of amniotic fluid demonstrable by amniocentesis.

Cervical mucus is a gel-like material that, after conception, undergoes a dramatic change under the influence of progesterone, which modifies both its appearance and physicochemical properties. The cervical mucus becomes thick, sticky, viscous, opaque, and gelatinous and forms a plug that obstructs the cervical canal. This plug appears in the cervical os as a clot of dense mucoid material. The cervical mucus plug is considered to be a physical barrier that prevents ascending infection by microorganisms located in the genital tract. The specific properties of this structure may play a key role in determining why some women develop an ascending intrauterine infection during pregnancy and others do not.

This study examined whether the cervical mucus plug exhibits inhibitory properties in vitro toward a selection of clinical isolates of gram-positive and gram-negative bacteria.

**Materials and methods**

The cervical mucus plug. The regional research ethics committee approved the project. Informed written and verbal consent was obtained from each patient.
Fifty-six cervical mucus plugs were shed spontaneously during labor. All were collected by the midwives digitally by vaginal exploration during active labor when cervical dilatation and the soft consistency of the cervical tissues made it easier to detect the cervical mucus plug as a separation tissue. Thirty-six were collected with intact membranes, and 20 were collected after a controlled or spontaneous rupture of the membranes. In 8 patients, the cervical mucus plugs were collected with forceps that were visually guided during vaginal inspection while the cervix was exposed with a speculum. In these cases, the cervical mucus plugs were gently removed from the cervical canal, allowing orientation of the uterine and vaginal end, respectively. Nearly all cervical mucus plugs were obtained intact, or in rare cases, in 2 pieces. Only intact cervical mucus plugs were used for experiments.

All the women were healthy, had had a normal pregnancy, and delivered normally at term. None of them had taken any systemic or local antibiotics before delivery. Each cervical mucus plug was inspected and weighed. For comparison with the cervical mucus plugs, we also obtained 9 samples of ovulatory cervical mucus that were collected on the day of oocyte aspiration from women attending the fertility clinic for in vitro fertilization. Cervical mucus was collected by aspiration with a 1-mL syringe from the cervical os.

All samples were stored at –70°C until analysis, except for 3 cervical mucus plugs; these plugs were initially divided into 2 pieces, and one piece was kept at 5°C and the other piece at –70°C, both for 1 hour, and thereafter used for the experiment.

**Microorganisms.** We used 8 representative bacterial isolates from the clinical laboratory as test agents for inhibition of bacterial growth in vitro. These included gram-positive *Streptococcus pyogenes*, 2 strains of *Streptococcus agalactiae*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Staphylococcus saprophyticus* and gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. The 2 strains of group B *Streptococcus* were a gift from J. M. Musser, Baylor College of Medicine, Houston, Texas, and were isolated in the United States of America: MMCA 3163 is a group B *Streptococcus* serotype III, high-virulence clonal, and MMCA 3164 is a group B *Streptococcus* serotype III, low-virulence clonal.

**Assay for antibacterial activity.** An agar overlay assay was used to test whether the cervical mucus plug inhibits bacterial growth within the agar. The bacteria were grown in transparent nutrient broth agar, and bacterial cultures were prepared by suspending bacteria grown overnight on 5% horse agar in sterile saline of McFarland No. 6 inoculum (10^9 mL). One mL was added to approximately 30 mL of melted nutrient agar maintained at 50°C and poured immediately into 9-cm Petri dishes (depth 5 mm).

The following numbers of plugs were used for experiments: (1) 21 intact cervical mucus plugs, each against 1 of the 8 bacterial strains; (2) 12 intact cervical mucus plugs, each against > 1 of the bacterial strains; and (3) 7 cervical mucus plugs separated into a vaginal and uterine part, against *Staphylococcus saprophyticus*. The cervical mucus plugs were placed on the bacterial suspension in agar, partly overlaying a cellulose nitrate filter (pore size, 0.45 µm) (Sartorius AG, Göttingen, Germany). The filter served as control for the occlusive effect of the cervical mucus plug. We tested 6 intact cervical mucus plugs with *Staphylococcus saprophyticus* as targets to determine whether the inhibition was blocked by filters, with different membrane pore sizes interposed between the plug and the agar surface (1, 2, 5, 10, 30, 50, 100, and 300 kd; Filtron Technology Corp, Northborough, Mass).

Finally, we compared ovulatory cervical mucus from 2 infertile women with 2 cervical mucus plugs from pregnant women with respect to growth-inhibitory activity against *Staphylococcus saprophyticus*. The ovulatory mucus and the cervical mucus plugs were placed on the same agar plate, both partly overlaying a cellulose nitrate filter.

The cervical mucus plugs were incubated at 37°C overnight. Inhibition of bacterial growth was defined as lack of bacterial growth under the plug and in a zone of more than 2 mm around the cervical mucus plug. The slices of the cervical mucus plug, the cervical mucus, and the filter were removed after 24 hours of incubation, and the agar plate was reincubated for another 24 hours to determine whether the inhibition of bacterial growth was reversible. After 24 and 48 hours, the plates were inspected for bacterial growth or inhibition. A microscope (×8 to ×20 magnification) was used to verify the observations. Growth of bacteria was seen as a distinctive haze in the agar, and inhibition of growth was defined as an absence of the haze under and around the cervical mucus plug, which was graded by a single observer into 3 classes: complete inhibition, partial inhibition, and no inhibition.

**Radial diffusion assay.** The agar radial diffusion assay was performed as described previously. Briefly, 8 cervical mucus plugs and 7 samples of ovulatory cervical mucus were tested, and overnight culture of group B *Streptococcus* and midlogarithmic growth phase *E. coli* were used to make the microbe-containing underlay.

Bacterial concentration was estimated photometrically at 620 nm for *E. coli* and group B *Streptococcus*. An OD_{620} reading of 0.2 corresponds to 10^7 colony-forming units/mL for the group B *Streptococcus* and 5 × 10^7 colony-forming units/mL for *E. coli*. Microbes were added to the 42°C underlay to a final concentration of 4 × 10^6 bacteria per 12-mL underlay just before pouring into a square Petri dish.

The underlay agar contained 1:100 dilution of trypticase soy broth and 1% agarose (Sigma, low EEO) in a cervical mucus plug buffer in which the composition of electrolytes mimics that of the cervical mucus plug: 150 mmol/L sodium chloride, 10 mmol/L potassium phosphate, and pH 4. Sixteen, evenly spaced, 3-mm wells were punched out in the solidified agar, and 5 µL of the cervical mucus plug or cervical mucus was pipetted into the
wells. Peptides were allowed to diffuse into the microbial agar at 37°C for 3 hours. To permit bacterial growth, nutrient agar containing 2:1 dilution of trypticase soy broth was poured on top of the bacterial lawn and, after it solidified, incubated inverted at 37°C overnight. Protegrin (1 µg/mL) was used as a positive control. For gentamicin, concentrations of 0.3, 0.15, 0.075, and 0.0375 µg/mL were used; and for E coli, concentrations of 2, 1, 0.5, and 0.25 µg/mL were used.

Antimicrobial activity was assessed by zones of clearance (no bacterial growth) around the wells. Radial diffusion units are defined as:

[Diameter of Clearing (mm) – Diameter of the Well (3 mm)] × 10.

Results

We found that the cervical mucus plug (Fig 1) at term is a well-defined, sticky or viscous (seldom watery) structure weighing between 3 and 18 grams. Macroscopically, it has a uterine part, which is sticky and clear, and a vaginal part, which is darker, more opaque, and firmer.

In initial studies, the cervical mucus plug exhibited antimicrobial activity against several strains of bacteria. Inhibition of bacterial growth was found both under and around the cervical mucus plug after 24 hours of incubation. After removal of the cervical mucus plug and reincubation for another 24 hours, no reversal of inhibition was seen. When tested against Staphylococcus saprophyticus, no difference was found in the antimicrobial activity between the vaginal and the uterine parts of the plugs or between fresh or frozen plugs. Comparison of the activity of cervical mucus plugs collected from 12 donors found variation in the antimicrobial spectrum and potency from donor to donor.

In the agar overlays, there was a complete zone of inhibition with Staphylococcus saprophyticus, E coli, and Pseudomonas aeruginosa; a partial zone with Enterococcus faecium, 1 of the 2 strains of Streptococcus agalactiae, Streptococcus pyogenes and Staphylococcus aureus; and a no inhibition zone with the other strain of Streptococcus agalactiae.

Whereas all the cervical mucus plugs showed inhibitory effect against Staphylococcus saprophyticus, Enterococcus faecium, E coli, and Pseudomonas aeruginosa, we found no inhibition by 4 of the plugs tested against Streptococcus agalactiae, Staphylococcus aureus, and Streptococcus pyogenes (Table).

The cervical mucus plugs tested against Staphylococcus saprophyticus inhibited its growth, but the 2 samples of ovulatory cervical mucus did not. The cervical mucus plug inhibited the growth of the bacteria by direct contact and also through membranes with pore sizes ranging from 2 kd up to 300 kd but not through membranes with a 1-kd cut-off. This indicates that the active factor in the cervical mucus plug may be as small as 2 kd. The inhibitory activity was not caused by the occlusion of the surface, because the filters alone had no inhibitory effect.

In the radial diffusion assay, we measured the antibacterial activity of the cervical mucus plug toward E coli and group B Streptococcus. The agar medium was made with the electrolyte composition of the cervical mucus plug, and the cervical mucus plug material was placed in each well.

After incubation overnight, a circular clearance zone appeared around the wells. All 8 of 8 cervical mucus plugs had a significant antibacterial activity toward group B Streptococcus, but only 2 of 8 cervical mucus plugs had antibacterial activity toward E coli.

The activities of the cervical mucus plugs against E coli had a median of zero radial diffusion units (interquartile range, 0-18.75), and for group B Streptococcus, the median was 52 radial diffusion units (interquartile range, 48-61) (Fig 2). We found a donor-to-donor variation in the radius of clearance among the donors (Fig 3) and a larger zone of clearance with the cervical mucus plug against group B Streptococcus (median, 75; interquartile range, 50-80) compared with samples of ovulatory cervical mucus (median, 40; interquartile range, 23-48; P = .019 by t test). No antimicrobial effect was seen when the wells were filled with distilled water as a control, and a clearance zone of 150 radial diffusion units was seen with protegrin at 1 µg/mL.

Comments

This is the first study to report the antibacterial property of the cervical mucus plug in pregnancy.

Previous studies have dealt with the antibacterial effect of cervical mucus from nonpregnant donors, and they
suggested that it was due to the presence of a lysozyme-like substance. Zuckerman et al found that the cervical mucus exerted a bactericidal effect during all phases of the menstrual cycle, though this was less pronounced at the time of ovulation. Recently, Eggert-Kruse et al have discovered that the cervical mucus has antibactericidal activity because, when they analyzed lysozyme in cervical mucus obtained during midcycle, they found a median concentration of 33 µg/mL. Chretien analyzed the ultrastructure and variations of human cervical mucus during pregnancy and menopause and proposed that the mucus forms a physical barrier in the uterine cervix, which prevents bacterial and fungal invasion from the vagina.

Antibacterial activity has been found in fetal membranes. In pregnancy, secretory leukocyte protease inhibitor (molecular mass, 11,700 d) is secreted from the epithelium lining the cervical crypts into the cervical mucus plug, where it is found in high concentrations. From the plug, the secretory leukocyte protease inhibitor diffuses to the fetal membranes, where it is also found in relatively high concentrations. Secretory leukocyte protease inhibitor has now been shown to have microbicidal properties.

This present study showed that material in intact cervical mucus plug is bactericidal and that it contains one or more diffusible substances active against bacteria. Studies with interposed filters suggest that the molecular mass of the active substance(s) is 1000 to 5000 d. Several substances previously described in epithelial secretions are candidates for this activity. Defensins are small (3 to 5 kd), antimicrobial cationic polypeptides. Two abundant subclasses of human defensins include the α-defensins HNP1-3, released from the neutrophil granulocytes, and the β-defensins HBD-1 and HBD-2, released from epithelial cells.

It could have been anticipated that the vaginal part of the plug, which is exposed to the bacteria-rich vaginal milieu, might have more active defense mechanisms than the uterine end. However, we found no difference in inhibitory activity between uterine and vaginal parts of the cervical mucus plug when using Staphylococcus saprophyticus as the test bacterium.

The cervical mucus plug, but not the ovulatory cervical mucus, was inhibitory against Staphylococcus saprophyticus; toward group B Streptococcus, the cervical mucus plug was nearly twice as inhibitory than the ovulatory cervical mucus. If further studies can confirm this finding, it could indicate that the antibacterial activity is augmented in pregnancy. The harvested cervical mucus plugs did not represent a random sampling of all deliveries, and it is possible that the ability to retrieve an intact cervical mucus plug from some patients but not others has an as-yet undetermined clinical significance.

The most prominent problem in obstetrics is preterm labor, which accounts for about half of all perinatal mor-

Fig 1. This photograph shows a typical cervical mucus plug from a normal delivery. The cervical mucus plug is placed randomly in the middle of a 9-cm Petri dish (depth 5 mm).
A large body of evidence suggests that perinatal infections may play an etiologic role in preterm rupture of the membranes and preterm labor. A large body of evidence suggests that perinatal infections may play an etiologic role in preterm rupture of the membranes and preterm labor. The antibacterial effect of cervical mucus plugs against a broad spectrum of gram-positive and gram-negative bacteria could play an important role in host defense against ascending infections in pregnancy. If so, individual variations in the potency and spectrum of this activity could predispose to ascending infections and its attendant complications. Thus, isolation and characterization of substance(s) responsible for this activity should be of much interest for future studies. High priority should also be placed on the analysis of differences in antibacterial activity of cervical mucus plugs from women with normal delivery and preterm delivery.

We thank Marlene Hylle and Lis Hyttel for excellent technical assistance and Hans Jakob Ingerslev and the
REFERENCES