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Negative cytology preceding cervical cancer: causes and prevention

I read with interest the article of Robertson and Woodend.¹ The authors reviewed 140 negative smears from 103 women who subsequently developed cervical cancer ("patients with microinvasive disease were excluded"). Forty eight smears (including one smear with severe inflammatory changes) were negative for abnormal cells (34.5%). As the authors consider of importance the "assessment of the quality of the smears received by a laboratory" it is surprising to read that the causes for true false negative smears were not considered.

True false negative smears may result from a series of reasons. Some may be avoided, provided that the patient, and the persons in charge of the collection of the material and of the staining of the smears, are well aware of the pitfalls of the method. In fact, the instrument used for the collection of the smears may in itself entrap atypical cells (figure 1), thus contributing to a false negative smear,² as the atypical cells collected from the area with cervical neoplasia may never reach the slide. The type of instrument used is also important; a significantly lower number of atypical cells are transferred to the slides by cotton swab applicators and plastic spatulas than by cervical brushes.³⁻⁵ The method by which the smear is handled also has an important role, and variables such as (a) the technique used to deposit the material on to the slide,⁶ (b) the pressure exerted when smearing the material,⁷ and (c) the quality of the cervical

mucus⁷ may also influence the presence of atypical cells in a cervical smear. Moreover, during staining procedures, detached material from the slides containing abnormal cells may render smears free from atypical cells, the result being a false negative report.⁸ Also, the detached material may become attached to other slides stained in the same batch (obtained from women without cervical neoplasias). The atypical cells attached to an "innocent" smear may yield a false positive smear.⁹

One other important factor which was not mentioned by Robertson and Woodend is the patient herself. In earlier investigations we showed that smears taken immediately before conisation in cases showing histopathological dysplasia or carcinoma in situ were often negative for atypical cells.¹⁰ Shortly before taking the smear, the cervico-vaginal area had been thoroughly disinfected with a cotton swab. The vigorous rubbing of the cervix in order to disinfect the surgical area may have removed the superficial layer of atypical cells that are usually collected by conventional techniques of cell sampling. A Swedish gynaecologist found that about one third of his patients had used manual washing deep into the vagina a few hours before examination. Such a washing should have the same effect as the "exfoliation" caused by disinfection before conisation. In some countries the use of vaginal douches is widespread. Thus the patient must be instructed to come to the gynaecologist "unprepared" for a cytological test. One other common finding in smears is the presence of well preserved spermatozoa, suggesting trauma of the cervical mucosa in the hours preceding cell collection (the absence of the spermatozoa, of course, does not rule out coitus). The effect of trauma in removing superficial layers of cervical epithelium has not been sufficiently emphasised.

It is obvious that factors other than the "screening fatigue" mentioned by Robertson and Woodend may be at stake, and that all participants in a cytological examination (the patient herself, midwives, staining tech-

nicians, screeners and (alas) even doctors) should receive the proper information and education, so they are aware that each step in the procedure is important to achieve good results.

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Drs Robertson and Woodend comment:

Dr Rubio draws attention to potential errors in the screening process mostly related to the taking of smears in the clinic. Our purpose was to deal mainly with the laboratory examination of smears. Dr Rubio is quite correct, however, to emphasise the importance of a good smear-taking technique and other pitfalls described in his many studies.

Cytotoxic activity of *Helicobacter pylori* enhanced by acetohydroxamic acid

One of the major pathogenicity factors described for *Helicobacter pylori* is its strong urease activity, which enables it to survive in the acid environment of the stomach. It has therefore been suggested that acetohydroxamic acid (AHA), a potent inhibitor of various bacterial ureases including *H. pylori* urease,¹ be incorporated into therapeutic regimens aimed at eradicating *H. pylori*. Synergistic effects between AHA and various antimicrobial agents against *H. pylori* in vitro have recently been reported by Phillips *et al* in this journal, making this approach even more attractive.² As we have observed that AHA enhances the cytotoxic effects produced by *H. pylori*, we would like to add a cautionary note to the discussion on the possible use of AHA for treatment of *H. pylori* infection as suggested by Mooney *et al*³ and again by Munster *et al*.⁴

We have been looking at the cytotoxic effects produced by supernatant fluids of *H. pylori* sonicates on HeLa cells in a quantitative microtitre cytotoxicity assay, performed in a modification of a test described by Gentry *et al*.⁵ Briefly, sterile filtered (0.45

Section of a wooden spatula after collection of cervical material before conisation for carcinoma in situ. Note a large number of normal and atypical squamous cells trapped in the wooden fibres of the spatula (toluidine blue stain).

Effect of acetohydroxamic acid (AHA) on cytotoxic activity of *H. pylori*: gel filtration fractions were added to HeLa cells either without or in the presence of 0.5 mg AHA/ml.

μ m) supernatant fluids (1 mg protein/ml) of sonicates from various *H. pylori* strains, obtained from endoscopy outpatients at the Department of Internal Medicine at the University Hospital Hamburg, were added in serial dilutions to 2×10^4 HeLa cells per well. After 48 hours of incubation photometric evaluation was carried out after staining residual cells with crystal violet. Results were expressed as CD50, defined as the highest titre of the sample leading to a 50% reduction of the optical density as compared with untreated cells. All 11 tested strains exhibited cytotoxic activity and yielded CD50s between 70 and 1867.

Gel filtration of sonicates using a sephacryl S 200 HR column ($n = 100$ cm, $r = 0.8$ cm; Pharmacia, Uppsala, Sweden) showed the cytotoxic fractions to be clearly distinct from the urease containing fractions.⁶ To gain further evidence for the independence of this cytotoxic effect from the urease activity, we used AHA (Sigma, St Louis, Missouri, USA) as a urease inhibitor. At a concentration of 0.5 mg/ml, we found the *H. pylori* urease activity to be effectively blocked (data not shown) as has been reported by Mobley *et al.*¹ While the addition of AHA to our cytotoxicity assay at the same concentration did not have an adverse effect on the HeLa cells when given either alone (control) or in combination with the urease fraction or a non-toxic fraction, combination of AHA with the cytotoxic fraction led to a sharp increase of cytotoxic activity (figure).

Thus AHA and some *H. pylori* cytotoxic factors apparently act synergistically on HeLa cells. So far, we do not know the basis for this synergism. Tentatively, one might speculate that *H. pylori* cytotoxin damages HeLa cell membranes, thus enabling AHA to accumulate intracellularly and then exert a cytotoxic effect. That hydroxamic acids actually have a cytotoxic potential in their own right on mammalian cells has been sufficiently demonstrated.^{7,8} In view of these findings we would therefore advise refraining from any clinical trials incorporating AHA into therapeutic regimens aimed at healing *H. pylori* induced gastritis, or even peptic ulcers, without further investigation of this synergistic effect.

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Dr Allardyce and Mr Bagshaw comment:

We agree with Hinrik von Wulffen and Thies Marquardt that clinical trials should be undertaken with caution and in the light of all relevant *in vitro* and *in vivo* experimental evidence. It is also true that the publications on *H. pylori* are littered with *in vitro* antibiotic data (including our own)¹ that may bear little relation to clinical outcome to the *in vivo* antibiotic functional efficacy *in vivo*.² There are, however, a number of points regarding results obtained using AHA in conjunction with *H. pylori* sonicates on HeLa cells *in vitro*. First, the results from Hamburg do not include dose or time response data. The synergistic antimicrobial effects reported by us were obtained using five to 10-fold less AHA. Also, the mean effects of continuous 48 hour exposure of HeLa cells to *H. pylori* sonicate fractions and AHA in culture are not accompanied by any indication of the variability of the observation or the length of time required for cellular damage to become irreparable. The issues of dose and timing are germane to the clinical finding that a single 750 mg dose of AHA inhibits *H. pylori* urease in infected patients by 86%.³ Second, there is a question relating to the practicality of the *in vitro* model using *H. pylori* sonicates on HeLa cells as opposed to intact organisms on gastric epithelial cells (preferably mucus secreting) and the absence of control sonicate sephacryl fractions from organisms other than *H. pylori*.

In the end, the clinical value of including urease inhibitors will rest on the balance between therapeutic efficacy and undesirable side effects. *In vitro* experiments, such as our own and those of von Wulffen and Marquardt, have some value by indicating general therapeutic directions and constraints. But that is all they can do. At present, what is needed are *in vivo Helicobacter* infection model experiments aimed at finding the most efficient means of eradicating *H. pylori* at the least cost to the wellbeing of the host.

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Alternative method for transporting and storing gastric biopsy cultures of *Helicobacter pylori*

Veenendal *et al* reported the use of sterile saline as a transport medium for gastric biopsy specimens in order to obtain adequate culture results for *Helicobacter pylori*.¹ Indeed, sterile saline is a simple and useful transport medium. Gastric biopsy specimens can, however, be put directly on to the surface of a suitable agar plate, thus avoiding the need for transport medium. This reduces costs, handling, and risk of contamination, which is the main problem in culturing *H. pylori*.

In our hospital the microbiology laboratory supplies the endoscopy unit with agar plates suitable for *H. pylori* culture. These are stored at 4°C in an ordinary freezer. Usually, our endoscopists take two gastric biopsy specimens with a sterilised forceps, and put them directly on to the surface of a suitable agar plate using a simple sterile needle, if necessary. The plate is subsequently stored in the freezer at 4°C until it is transferred to the microbiology laboratory. In our experience over 10 months, the specimens stored on agar plates may remain suitable for culture for at least seven days at 4°C (from seven to 14 days), and for at least 25 hours at room temperature (from 25 to 28 hours). Samples can therefore be collected, even from distant endoscopy units once or twice a week.

According to Veenendal *et al*, one antral biopsy specimen is adequate for culture. In our experience this holds true except in cases of previous omeprazole treatment, or where there is a strong reduction of parietal cells. In these circumstances *H. pylori* may be present only in the oxyntic mucosa.^{2,3} This may be due to the change in antral pH from acid to neutral, whereas a scanty local excretion of acid still present in the fundic area may permit *H. pylori* survival in this zone.^{2,4,5} A false negative culture of a single antral specimen may also be due to an extensive intestinal metaplasia of the antral mucosa.⁶

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