Isolation of Stool-Derived Mucus Provides a High Yield of Colonocytes Suitable for Early Detection of Colorectal Carcinoma

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Abstract

Background: Promising stool-based screening tests for colorectal carcinoma (CRC) rely on detection of exfoliated colonocytes or their contents. However, methods of colonocyte retrieval have not been studied systematically and current approaches are restricted by low yields. We examined colonocyte numbers in stool wash fractions and assessed the suitability of retrieved cells for immunocytochemistry for minichromosome maintenance protein 2 (MCM2), a marker of the proliferative deregulation that characterizes malignancy.

Methods: Colonocyte numbers were accurately quantified in 129 wash fractions derived from 18 stools, comparing the mucus retained by a 125-μm filter (F fraction) with the fine and coarse content in the filtrate (S and P fractions, respectively). MCM2 immunocytochemistry was done on sections of fibrin clot containing filter-derived mucus, obtained from stools of eight independent subjects.

Results: Total colonocyte yield in the F fraction (mean, 433.8 per 100 μL) was higher than in the S (140.3) and P (204.6) fractions (P = 0.004 and 0.03, respectively) due to increased numbers of morphologically abnormal cells, which predominantly represented malignant cells in samples from CRC patients. Several thousand abnormal cells could be obtained from stool-derived mucus in all CRC patients, an order of magnitude greater than numbers in subjects without CRC. Median MCM2 labeling index in abnormal cells was 50% (range, 30-60%) in CRC patients and 0% in subjects without CRC. Cells in clot sections were well preserved and not obscured by fecal debris.

Conclusions: Isolation of stool-derived mucus is technically straightforward and can improve the performance of protein-based and/or nucleic acid–based approaches to CRC screening. (Cancer Epidemiol Biomarkers Prev 2009;18(7):2006–13)

Introduction

Although colorectal carcinoma (CRC) is amenable to prevention by population screening (1), currently available tests are of limited value. Colonoscopy, flexible sigmoidoscopy, barium enemas, and virtual colonography are invasive and costly and may cause morbidity, including colonic perforation. Noninvasive screening tests based on stool offer advantages of simplicity, low cost, and patient acceptability. The most commonly used is fecal occult blood testing (FOBT), which is restricted by relatively low sensitivity at assay settings that provide acceptable levels of specificity (1-3). Stool tests based on isolated colonocytes rather than blood may offer improved performance, when used either in isolation or in combination, including with FOBT.

Various approaches to colonocyte-based screening are available, including an extensive battery of DNA tests and detection of proteins such as minichromosome maintenance (MCM) proteins (1). The latter are sensitive and specific indicators of cell cycle entry and useful markers of the proliferative deregulation that characterizes malignancy and premalignancy (4). Aberrant expression of MCMs has been exploited to improve the detection of several common cancers in at-risk populations. Our group previously proved the principle that expression of MCMs can discriminate between normal and malignant colonocytes isolated from stool (5). As proliferative deregulation represents the convergence point of all cell growth signaling abnormalities in malignancy, MCM detection is a simpler and cheaper alternative to the multitarget DNA assays needed to identify CRC mutations.

The effectiveness of colonocyte-based screening methods is likely to depend on the number of cells that can be isolated from stool samples. Ideally, techniques for colonocyte isolation need to maximize cell retrieval while minimizing the amount of background fecal debris, which can obscure cellular morphology and inhibit screening assays (6, 7). Several groups have attempted to isolate exfoliated colonocytes from stool, either from homogenized samples or from the stool surface, using density gradient centrifugation–based and/or immunomagnetic bead–based separation (reviewed in ref. 8). However, cell yields are generally very low, often with
conspicuous background debris, which makes cell identification difficult and time consuming. Consequently, such preparations would be unsuitable for high-throughput population screening programs.

In view of these issues, we sought to develop a method of increasing the numbers of colonocytes retrieved from stool. We systematically examined the numbers of cells that can be retrieved from different isolation fractions obtained by filtration of stool washings. We were particularly interested in obtaining colonic mucus, as we considered that its protective properties might create a niche in which colonocytes would be preserved in relative abundance at the stool surface (8, 9). We further investigated whether retrieved colonocytes were suitable for MCM2 immunocytochemistry, thereby enabling at least one strategy for improved population screening for CRC.

Materials and Methods

Patient Details and Study Overview. Approval for the study was granted from the Cambridge Local Research Ethics Committee (Ref: 00/385). Multiple stool samples were obtained from subjects attending the colorectal clinic at Addenbrooke's Hospital Cambridge with symptoms suggestive of CRC. The first, and principal, component of the study was an examination of colonocyte yield and background debris in different fractions isolated from stool. This involved detailed analysis of 129 preparations, generated from 18 stools provided by five patients (subjects A.1-A.5; Table 1). Four of these patients were subsequently found by clinical investigations to have left-sided CRC (i.e., arising distal to the splenic flexure), whereas the fifth was found to have a normal large bowel (Table 1). Patient A.4 underwent preoperative radiotherapy: stool samples were obtained before this commencing.

The second component of the study was to investigate the suitability of colonocytes retrieved from one stool fraction (the F fraction, containing the colonic mucus) for immunocytochemical detection of MCM proteins. For this, we examined eight stool samples, each from a different individual (subjects B.1-B.8; Table 1). Six samples were obtained before surgical resection from patients known to have CRC (subjects B.1-B.6). In five of these patients, the CRC was left sided, whereas in the sixth it affected the transverse colon. The other two stool samples were from normal volunteers (subjects B.7 and B.8).

Stool Procurement and Processing for Assessment of Cell Yield. Subjects were requested to defecate into a clear colorless plastic bag attached to a simple metal frame placed on the toilet rim at home. The samples were placed onto crushed ice and processed within 2 h. For the study assessing colonocyte yield and background debris, we used the protocol summarized in Fig. 1. First, each sample was weighed and its consistency was assessed by one observer (V.W.). Four categories of consistency were defined: “hard” (multiple pellets that do not deform with handling), “firm” (the stool has clear lines of natural cleavage, maintains its shape during washing, but deforms with minimal handling), and “loose” (semisolid).

Stools were washed in PBS containing ~0.5% (w/v) of the mucolytic agent ammonium thioglycolate (Sigma-Aldrich) using 1 mL/g of stool. The bag was manually supported and the stool was gently agitated until the surface layers, to a depth of approximately 2 to 3 mm, were liberated. The time required was 20 s to 2 min depending on stool consistency, harder stools being washed for longer. The stool wash was then centrifuged at 800 rpm for 5 min, and the supernatant (in which colonocytes were not seen; data not shown) was discarded.

To optimize cell preservation for subsequent assessment of cell yield, the pellet was then fixed by resuspension for 45 min in CytoLyt cytologic preservation solution (Cytyc Corp.) using 5 mL/g of the original stool. All washing steps required to generate the three fractions examined (see below) were also done using this solution, with the aim of optimizing cell morphology before cytologic assessment.

The resuspended pellet was filtered through a brass frame holding a 125-μm stainless steel mesh (SV0923; Endecotts Ltd.) into a glass beaker, enabling isolation of the three fractions analyzed. The first fraction represented the solids retained by the filter, which included the colonic mucus (the F fraction). The other two fractions were derived from the filtrate following light centrifugation and represented the finer content in the supernatant (the S fraction) and the coarser content in the pellet (the P fraction).

To generate the F fraction, the mesh was inverted and the filter solids were retrieved by pouring 100 to 300 mL of wash solution from a height of up to 20 cm. After centrifugation at 800 rpm for 5 min, the supernatant was discarded and the mucus (visible as pale yellow/brown adherent strands) was retrieved from the pellets using a 200 μL pipette tip. The mucus was then placed directly onto a silane-coated glass microscopy slide and smeared until the whole surface was thinly covered, a procedure that required ~100 μL of mucus. The slides were sprayed with Cytofix (Surgipath Ltd.) and allowed to air dry. Although the amount of mucus that could be retrieved from each stool was variable, it was possible to prepare two to six F fraction slides from each of the 18 stool samples processed.

To generate the P and S fractions, fecal solids in the filtrate were first sedimented at 400 rpm for 5 min. The relatively coarse material in the pellet was resuspended using 1 mL/g of the original stool weight, followed by repelleting at 800 rpm for 5 min, to generate the P fraction. The relatively fine content in the supernatant from the 400 rpm sedimentation of the filtrate was pelleted at 800 rpm for 5 min to generate the S fraction. For both the S and P fractions, centrifugation at speeds >800 rpm caused cell fragmentation (data not shown). Aliquots (100 μL) of the S and P fraction pellets were placed directly onto microscope slides, sprayed with Cytofix, and allowed to air dry.

Assessment of Cell Yield. For each stool sample, at least two slides prepared from the F, S, and P fractions were stained by H&E for assessment of cell yield. Every slide was assessed individually and contributed independent data for statistical analysis. All colonocytes
identified on each slide were counted to produce a “total cell” count. Squamous and mononuclear cells were excluded. Each colonocyte count was also divided into the number of cells that seemed morphologically normal using standard criteria (10) and the number that did not, categories that were referred to as “normal” and “other” colonocytes, respectively. The latter group represented colonocytes showing any deviation from normal morphology. In the patients with CRC, the majority of these were frankly malignant. However, there were also cells [seen in the sample from the subject with normal large bowel (A.5) as well as those from CRC patients] that were atypical without showing features of malignancy, for example, representing colonocytes in the early stages of cell death. Fragmented nuclei were not counted. Small macrophages resembling colonocytes may also have been scored as other cells on occasions, although we estimate, based on morphologic criteria, that these represented <2% of the other cells.

In all, colonocyte numbers were determined on 129 slides. All were assessed by a single individual (V.W.), who obtained ~3 y of experience of identifying colonocytes in stool preparations. Slides from the F and S fractions were screened blind to the fraction type, although this was not possible for the F fraction because of the presence of mucus. To allow for variations in stool weight, an equivalent number of slides from each of the three fractions was assessed for each stool sample. In addition, the aliquots taken from each fraction were always of the same volume, 100 μL, the volume required to cover the surface of a standard microscope slide.

Assessment of Background Fecal Debris/Slide “Cleanliness”. All slides were also assessed by one observer (V.W.) for the amount of background fecal debris, representing plant and bacterial cells and other nonspecific particulate matter. Four categories of slide cleanliness were defined: very clean (minimal or no background debris), clean (background debris present but finely dispersed or localized in clumps), dirty (a widespread “film” of background debris, of variable thickness), and very dirty (a “blanket” of background fecal debris obscuring most fields).

Statistical Analysis of Cell Yield and Background Debris. Cell yield distributions for different isolation fractions and stool consistencies were compared using rank-sum nonparametric tests (Kruskal-Wallis for multiple comparisons and Wilcoxon/Mann-Whitney for pairwise comparison). To strengthen the statistical reliability, ANOVA (multiple comparison) and t test (pairwise comparison) were applied to compare distributions of log10(CY + 1), where CY = cell yield. This correction of cell yield aimed to ensure the normalcy of distributions without affecting the biological validity of the comparisons. Significance levels obtained with nonparametric and parametric tests were generally consistent. One-tail or two-tail tests were applied according to the reasonable alternative hypothesis. Slide cleanliness levels were compared using both contingency tables (CT) and the described rank-sum tests, applying a scoring system of 1 = very dirty, 2 = dirty, 3 = clean, and 4 = very clean. For all statistical tests, differences were considered significant if P < 0.05.

Immunocytochemistry for MCM2 in Mucus-Derived Colonocytes. In the second component of the study, we assessed whether the colonocytes in filter-retained mucus from CRC patients could be stained for MCM2 and whether a test combining mucus retrieval and MCM2 immunocytochemistry could discriminate between CRC patients and normal volunteers. For this component of the study, the mucus was suspended in a plasma clot followed by cutting of 5-μm sections for immunocytochemistry (see Results for justification of this approach). Moreover, as the principal aim was now to analyze the presence or absence of immunopositive nuclei, and not to do the detailed morphologic assessment needed for cell yield quantification, the method for isolating the F fraction was simplified. Rather than fixing the pelleted washings, they were resuspended in 50 mL PBS and filtered immediately. We observed that this step made the mucus less dense and viscous but did not affect its retention by the filter (data not shown). All subsequent washes were in PBS rather than CytoLyt.

The retrieved filter solids were sedimented at 1,600 rpm for 10 min. The mucus (generally amounting to

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**Table 1. Clinicopathologic details of patients providing stool samples**

<table>
<thead>
<tr>
<th>Study group</th>
<th>Patient ID</th>
<th>Age</th>
<th>Sex</th>
<th>Site of CRC</th>
<th>Maximum dimension (cm)</th>
<th>Histologic differentiation*</th>
<th>Dukes’ stage</th>
<th>TNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonocyte yield</td>
<td>A.1</td>
<td>50</td>
<td>F</td>
<td>Rectum</td>
<td>2.5</td>
<td>Moderate</td>
<td>A</td>
<td>pT2, N0, Mx</td>
</tr>
<tr>
<td></td>
<td>A.2</td>
<td>69</td>
<td>M</td>
<td>Recto-sigmoid</td>
<td>3.5</td>
<td>Moderate</td>
<td>C1</td>
<td>pT3, N1, Mx</td>
</tr>
<tr>
<td></td>
<td>A.3</td>
<td>84</td>
<td>F</td>
<td>Rectum</td>
<td>3</td>
<td>Moderate</td>
<td>D</td>
<td>pT3, N2, M1</td>
</tr>
<tr>
<td></td>
<td>A.4</td>
<td>75</td>
<td>M</td>
<td>Rectum</td>
<td>3</td>
<td>Moderate</td>
<td>A</td>
<td>ypT2, yN0, yMx</td>
</tr>
<tr>
<td></td>
<td>A.5</td>
<td>66</td>
<td>M</td>
<td>No CRC</td>
<td>5</td>
<td>Well</td>
<td>B</td>
<td>pT2, N0, Mx</td>
</tr>
<tr>
<td></td>
<td>B.1</td>
<td>79</td>
<td>F</td>
<td>Transverse</td>
<td>5</td>
<td>Moderate</td>
<td>C1</td>
<td>pT3, N2, Mx</td>
</tr>
<tr>
<td></td>
<td>B.2</td>
<td>55</td>
<td>M</td>
<td>Rectum</td>
<td>10</td>
<td>Poor</td>
<td>C1</td>
<td>pT3, N1, Mx</td>
</tr>
<tr>
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<td>73</td>
<td>M</td>
<td>Rectum</td>
<td>2.5</td>
<td>Moderate</td>
<td>B</td>
<td>pT3, N0, Mx</td>
</tr>
<tr>
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<td>B.4</td>
<td>78</td>
<td>F</td>
<td>Sigmoid</td>
<td>5</td>
<td>Moderate</td>
<td>B</td>
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<tr>
<td></td>
<td>B.5</td>
<td>69</td>
<td>M</td>
<td>Rectum</td>
<td>3.5</td>
<td>Moderate</td>
<td>B</td>
<td>pT3, N0, Mx</td>
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<tr>
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<td>B.7</td>
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<td>M</td>
<td>No CRC</td>
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<td>Moderate</td>
<td>B</td>
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</tr>
<tr>
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<td>B.8</td>
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<td>F</td>
<td>No CRC</td>
<td>5</td>
<td>Moderate</td>
<td>B</td>
<td>pT3, N0, Mx</td>
</tr>
</tbody>
</table>

NOTE: The prefix A in the patient identifier column denotes the patients who provided multiple samples for the detailed cell yield analysis, whereas the prefix B denotes patients who provided a single sample for MCM2 immunocytochemistry. Abbreviation: TNM, tumor-node-metastasis staging.

*All tumors were adenocarcinomas.
200-500 μL in total) was transferred to a 1.5 mL test tube and compacted by centrifugation at 1,200 rpm for 1 min (producing volumes of ~100-300 μL). The clot was prepared by adding 200 μL of human plasma (Department of Haematology, Addenbrooke’s Hospital) followed by bovine thrombin (Diagnostic Reagents Ltd.) in 20 μL aliquots until clotting and contraction occurred (generally after addition of 40 μL thrombin).

The entire clot (volume, 100-250 μL) was wrapped in cellulose paper, placed in a histology cassette, and fixed for 12 to 24 h in neutral buffered formalin. The maximum dimension of the fixed clot ranged from 10 to 25 mm. The clot was then processed to paraffin and 5-μm sections were cut onto silane-coated slides, placing three sections on each slide. Antigen retrieval and immunocytochemistry were carried out with in-house mouse monoclonal primary antibodies against MCM2 (at 1:40) using conditions similar to those devised previously for detection of abnormal cells in cervical smears (11-13).

Stained slides were examined by a single individual (E.I.), who had been trained by a senior researcher with over 20 y of experience of examining immunocytochemical preparations (C.S.). The observer had ~12 mo of experience of identifying colonocytes and immunostained cells in stool preparations. For all samples, 10 or more slides (range, 10-59) were stained by H&E to allow assessment of the number of colonocytes per slide that were not morphologically normal (corresponding to other cells in the cell yield study). Cells were quantified using a categorical approach rather than the precise counting used in the cell yield study. Based on the spread of counts determined in preliminary analyses, the categories selected were as follows: no cells, <10, 10 to 20, 20 to 40, and >40. A further 10 slides from each sample were stained by immunocytochemistry to determine the percentage of abnormal colonocytes with detectable MCM2, estimated to the nearest 10%.

**Results**

**Colonocyte Retrieval Study.** In total, 129 slides underwent detailed quantitative assessment of colonocyte yield. The slides were generated from 54 fractions, obtained from 18 stools (median weight, 30 g) provided by the five patients in the cell yield study. Stool consistency ranged from soft to hard, with no stool being loose. Although the amount of mucus that could be retrieved from each stool was variable, at least two F fraction slides could be obtained from each stool processed. Colonocytes were usually seen in all fractions assessed. Examples of cells in the F fraction are shown in Fig. 2. In patients with CRC, the majority of other cells were frankly malignant, including occasional large papillary clusters (Fig. 2D).

**The F Fraction Provides the Highest Yield of Colonocytes.** When assessing samples from all five subjects, we observed a significant difference in the total cell counts across the three fractions ($P = 0.011$, Kruskall-Wallis; $P = 0.008$, ANOVA; Fig. 3A). This was explained by significantly higher counts in the F fraction versus the P fraction ($H_1: P < F$, $P = 0.02$, Wilcoxon; $H_1: P < F$, $P = 0.03$, t test) and in the F fraction versus the S fraction ($H_1: S < F$, $P = 0.002$, Wilcoxon; $H_1: S < F$, $P = 0.004$, t test; Fig. 3A). There was no significant difference between the P and S fractions ($H_1: P = S$, $P = 0.3$, Wilcoxon; $H_1: S = F$, $P = 0.4$, t test). When analyzing data from all samples, cell yields per slide (i.e., per 100 μL sample) were around 2- to 3-fold higher in the F fraction (mean, 433.8; median, 151.5) than in the P fraction (mean, 204.6; median, 79.5) and the S fraction (mean, 140.3; median, 40). All values observed were an order of magnitude higher than those seen previously by us using stool smears and magnetic microbead retrieval (5). It should be noted, however, that there was substantial variation between cell counts in different slides obtained from the same individual and

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**Figure 1.** Summary of protocol used to generate the F, P, and S fractions from stool washings.
also from the same stool (see Fig. 3, where data from individual patients are color coded).

For all fractions, the lowest total cell counts were seen for the patient without CRC (Fig. 3A). In this individual (A.5), the median numbers of cells per slide were 23.5 (F fraction), 7.5 (P fraction), and 3.5 (S fraction) compared with equivalent values for the four CRC patients of 280.0 (F), 121.5 (P), and 87.0 (S). For the four CRC patients, we still observed significantly higher counts in the F fraction versus the P fraction (H1: P < F, P = 0.009, Wilcoxon; H1: P < F, P = 0.02, t test) and in the F fraction versus the S fraction (H1: S < F, P = 0.01, Wilcoxon; H1: S < F, P = 0.002, t test), with no significant difference between the P and S fractions (H1: P = S, P = 0.4, Wilcoxon; H1: S = F, P = 0.4, t test).

Interestingly, there was no significant difference in yield of normal colonocytes between the three fractions when analyzing all five patients (P = 0.86, Kruskall-Wallis; P = 0.999, ANOVA; Fig. 3B) or the four with CRC (P = 0.89, Kruskall-Wallis; P = 0.60, ANOVA). Pairwise comparisons of the yield of normal colonocytes between the fractions also showed no significant differences. In contrast, the yield of other cells did show a significant difference between the fractions when analyzing all five patients (P = 0.02, Kruskall-Wallis; P = 0.003, ANOVA; Fig. 3C) or the four with CRC (P = 0.002, Kruskall-Wallis; P = 0.0006, ANOVA). This difference was explained by significantly higher yields in the F fraction versus the P fraction (for all five patients H1: P < F, P = 0.03, Wilcoxon; H1: P < F, P = 0.03, t test) and in the F fraction versus the S fraction (for all five patients H1: S < F, P = 0.003, Wilcoxon; H1: S < F, P = 0.003, t test; Fig. 3C). There was no significant difference between the P and S fractions (for all five patients H1: P = S, P = 0.3, Wilcoxon; H1: S = F, P = 0.3, t test). For the four CRC patients only, the difference in yield of other cells between the F fraction and the P and S fractions was even more striking (H1: P < F, P = 0.009, Wilcoxon; H1: P < F, P = 0.02, t test; H1: S < F, P = 0.0005, Wilcoxon; H1: S < F, P = 0.0009, t test).

Again, no significant difference between the P and S fractions was detected (H1: P = S, P = 0.2, Wilcoxon; H1: S = F, P = 0.2, t test).

**Cell Yield Increases with Harder Stool Consistency.** When analyzing samples from all five patients, there was no significant association between cell yield and stool consistency for any of the fractions individually (F: P = 0.09, Kruskall-Wallis; F: P = 0.2, ANOVA; P: P = 0.1, Kruskall-Wallis; P: P = 0.06, ANOVA; S: P = 0.2, Kruskall-Wallis; S: P = 0.08, ANOVA; Supplementary Fig. S1), although cell yield did vary significantly with stool consistency when taking all fractions together (P = 0.009, Kruskall-Wallis; P = 0.002, ANOVA). The yield from soft stools was significantly less than from firm stools (H1: S < F, P = 0.001, Wilcoxon; H1: S < F, P = 0.001, t test) and hard stools (H1: S < H, P = 0.03, Wilcoxon; H1: S < H, P = 0.03, t test), although there was no significant difference in yield between firm and hard stools (H1: F = H, P = 0.7, t test).

**The F Fraction Shows a Reduction in Slide Cleanliness.** When assessing all samples, we observed variation in slide cleanliness (reflecting background fecal debris) across the three fractions (P = 0.0004, Kruskall-Wallis; P = 0.00015, CT). The mean cleanliness index in the F fraction (2.6) was reduced compared with the P fraction.
and the S fraction (3.3; \( F \neq S: P = 0.009, \text{CT} \); \( F < S: P = 0.001, \text{Wilcoxon} \)). This was due to contamination of the mucus in some preparations by adherent coarse fecal aggregates. There was no significant difference in slide cleanliness between the P and S fractions (\( F \neq S: P = 0.1144, \text{CT} \); \( F < S: P = 0.1, \text{Wilcoxon} \)). For the F fraction, harder stools showed increased cleanliness (\( P = 0.0001, \text{Kruskall-Wallis} \); \( P = 0.0000005, \text{CT} \)).

Abnormal Colonocytes in Mucus Can Be Labeled by MCM2 Immunocytochemistry. We next tested whether colonocytes in filter-retained mucus were suitable for MCM immunocytochemistry. In preliminary work, we observed that the F fraction was not suited to conventional liquid-based cytology using ThinPrep (Cytyc) or SurePath (BD-TriPath) systems, as the mucus impaired sample processing, did not produce monolayers (a requirement for automated slide assessment), and did not adhere adequately to microscopy slides (data not shown). Such limitations could not be overcome by further mucolytic treatment using acetylcysteine (0.1 mg/mL) or Mucolexx (Shandon; 50% v/v). We therefore used an alternative approach in which the mucus was suspended in a fibrin clot followed by immunocytochemical assessment of cut sections.

To establish the number of slides that could be generated from a paraffin-embedded plasma clot, four blocks of clots from CRC patients were cut through. With three sections per slide, the median number of slides produced was 106 (range, 93-118). For all eight blocks examined (six from CRC patients and two from normal volunteers), all sections adhered well to the glass slides during H&E and immunocytochemical staining. Cell morphology was considerably easier to assess in the clot sections than in the direct preparations of mucus used for the preceding cell yield study. Moreover, fecal debris cut at 5 \( \mu \)m did not obscure cell morphology as much as the coarse aggregates encountered on the direct preparations.

For the blocks from CRC patients, morphologically abnormal colonocytes were seen on all slides examined (Fig. 2E). The numbers of morphologically abnormal cells per slide generally varied from <10 to >40, although for one patient (B.2) many slides contained several hundred cells. Based on the amount of sections that could be obtained from each block, we estimated the number of abnormal cells present in the mucus-containing clots from the six CRC patients to be approximately 2,000 to 6,000, consistent with counts seen in the F fraction slides from patients A.1 to A.4. In contrast, morphologically abnormal cells were rare in the samples from the two volunteers (B.7 and B.8), with <10 or 0 cells per slide.

Many of the mucus-derived abnormal colonocytes from CRC patients expressed MCM2. Immunolabeled cells could be identified readily even at low magnification (Fig. 2F and G). The frequency of staining was consistent between slides and between the three sections on each slide. For the six CRC patients, the median frequency of MCM2 expression in the morphologically abnormal colonocytes was 50% (range, 30-60%). Cells from the patient with the more proximal CRC (B.1) did not show a relatively low frequency of MCM2 expression (50%). For each of the two normal volunteers, no MCM-positive cells were seen in any of the 10 slides examined.

Discussion

Although cell exfoliation is an important mechanism for regulating colonocyte numbers, the amount of shedding...
from normal large bowel mucosa is thought to be relatively low (8). In contrast, disruption of cell death and adhesion in malignancy (14, 15) dramatically increases exfoliation, leading to accumulation of malignant cells in the mucus layer overlying CRCs (3, 9, 16).

We report here that stool-derived mucus, which can be retrieved using a 125-μm filter, represents the stool wash fraction that contains the greatest number of colonocytes per 100 μL aliquot, the volume required to cover a standard microscope slide. Of importance to screening applications, the increased yield is due to enrichment for morphologically atypical colonocytes, which predominantly represent malignant cells in patients with CRC. Colonc mucus, which is often produced in abundance by CRCs (9, 17), may aid preservation of exfoliated colonocytes, both in the large bowel lumen and during the colonocyte isolation procedure, by maintaining osmotic balance and protecting against lysis by bacterial proteases and toxins (3, 9).

Evidence is emerging that mucus and its cellular cargo are able to migrate distally in the colon independent of stool (8, 18). In consequence, although the mucus present on a stool surface after defecation may have been picked up in the distal large bowel, it may nevertheless include cells derived from the entire colorectal mucosa. This would explain why malignant cells from cecal and other right-sided CRC can be isolated from stool after defecation (5) despite the luminal contents generally being liquid in the most proximal large bowel. Although procedures are available for procuring rectal mucus directly, such as luminal balloon inflation (8, 19), isolation of mucus from stool is a noninvasive approach that offers potential advantages of cost and patient acceptability.

We retrieved several thousand atypical cells per stool sample from the CRC patients in our study (the majority of whom had left-sided tumors). This was a substantially greater number than we previously observed using smears and magnetic microbeads (5). It is likely that other groups also obtained low yields using density gradients or microbeads, considering the available images and the lack of cell quantification data in most published studies (9, 20-23). Our method also allowed us to obtain morphologically atypical cells from all 20 stools from CRC patients, including the subject with transverse colon malignancy. These data contrast with findings using homogenized stool samples, which in one study yielded atypical cells in only 28% of CRC patients (albeit with a superior sample size of 116; ref. 21).

Future studies need to investigate the numbers of atypical cells (as well as numbers of MCM-positive cells) in stool-derived mucus from an adequately powered set of patients with and without CRC and analyze associations with tumor variables such as site, size, grade, and stage. Nevertheless, our present data argue that stool-derived mucus would be the most appropriate fraction to use in clinical evaluation studies of colonocyte-based CRC screening tests. The approach may also be applicable to early detection of adenomas (1). In our view, retrieval of cells from an adenoma is likely to depend on its size and site, with the probability of success being greatest for large, distal lesions. However, this is an area that again requires systematic study in an appropriately sized group.

In our experiments, mucus could be retrieved from all 26 stools processed. The relatively low concentration of the mucolytic agent thioglycolate in our stool wash solution (~0.5%; compared with >5% in previous studies; refs. 5, 20, 24) may have increased overall cell yield by being adequate to liberate mucus from stool but insufficient to produce substantial mucolysis and colonocyte liberation before filtration and mucus retrieval.

We found that stools of a harder consistency produced significantly greater colonocyte yield, as well as a tendency to increased cleanliness. One factor in these observations is that the time for initial stool washing can be extended for hard stools before there is overt contamination of washings with fecal material. Harder stools may also stimulate mucus production and directly increase colonocyte release through exfoliation (3). They may also allow more mucus to be released for a given weight of stool, as pockets of mucus are frequently retained between individual pellets of hard stool.

We further showed that the colonocytes in stool-derived mucus are suitable for detection of MCMs by immunocytochemistry. This is an encouraging observation, as it suggests that combination of mucus retrieval and MCM detection may be applicable in population screening for CRC. As mucus does not lend itself to LBC processing, there is a requirement for extra handling steps in preparing sections of mucus-containing clot. However, the additional procedures needed are rapid and technologically straightforward, requiring skills that are already available in any routine histopathology/cytopathology laboratory, and which could be applied in a high-throughput setting. Moreover, an important practical advantage of the clot approach is that cell morphology is very clearly seen using H&E or immunocytochemistry preparations and is not obscured by fecal debris. This should lead to a reduction in the amount of time required to analyze slides and therefore greater throughput and cost-effectiveness.

Colonocyte preparations may also add value to other strategies for early detection of CRC. The good cell morphology seen in clot sections suggests that DNA is likely to be well preserved, which may increase the effectiveness of DNA-based tests, whereas the relatively high yield of cells in stool-derived mucus suggests that multiple tests could be done. Unlike cell isolation methods using density gradients or immunomagnetic beads (8, 20, 22, 23, 25), we observed comparatively few squamous cells in our stool-derived mucus samples, indicating that the nucleic acids isolated would largely be colonocyte derived and not substantially diluted by normal DNA from squamous cells. Moreover, the relative representation of fecal matter in the mucus fraction is far less than in stool homogenates. Although colonocyte-based assays requiring whole stool samples may not be appropriate for first-line population screening, they may of value as reflex tests after a positive FOBT. Should such tests improve the specificity of the FOBT, it might be possible to modify the variables of the latter test to allow greater sensitivity for disease in the first screening round.

In conclusion, we have shown that colorectal mucus derived from stool provides greater yields of colonocytes in patients with CRC and is particularly enriched for a population of morphologically abnormal cells that predominantly represent malignant cells in CRC patients. It is possible to isolate such cells from stool washings using a simple, rapid method that does not
require specialist skills and is suitable for standardization and high-throughput use. The cells isolated are well preserved and appropriate for CRC screening using MCM immunocytochemistry and, potentially, nucleic acid–based approaches.

**Disclosure of Potential Conflicts of Interest**

N. Coleman and R.A. Laskey are entitled to a share of royalties received by Cancer Research Technology Ltd. on sales of products related to the use of MCM detection in cancer diagnosis.

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**References**

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