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Improved cervical smear assessment using antibodies against proteins that regulate DNA replication

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ABSTRACT **Carcinoma of the cervix is one of the most common malignancies. Papanicolaou (Pap) smear tests have reduced mortality by up to 70%. Nevertheless their interpretation is notoriously difficult with high false-negative rates and frequently fatal consequences. We have addressed this problem by using affinity-purified antibodies against human proteins that regulate DNA replication, namely Cdc6 and Mcm5. These antibodies were applied to sections and smears of normal and diseased uterine cervix by using immunoperoxidase or immunofluorescence to detect abnormal precursor malignant cells. Antibodies against Cdc6 and Mcm5 stain abnormal cells in cervical smears and sections with remarkably high specificity and sensitivity. Proliferation markers Ki-67 and proliferating cell nuclear antigen are much less effective. The majority of abnormal precursor malignant cells are stained in both low-grade and high-grade squamous intraepithelial lesions. Immunostaining of cervical smears can be combined with the conventional Pap stain so that all the morphological information from the conventional method is conserved. Thus antibodies against proteins that regulate DNA replication can reduce the high false-negative rate of the Pap smear test and may facilitate mass automated screening.**

Despite an intensive and expensive screening program, carcinoma of the cervix is the eighth most common malignancy of women in the United Kingdom and the most common malignancy in women under 35 years of age (1). In the developing world it is the most common malignancy in women between the ages of 35–45 years with an estimated 437,000 new cases each year (2).

The majority of cases represent squamous cell carcinoma and are strongly associated with infection by high-risk types of human papilloma virus (HPV), such as 16, 18, and 31 (3). Cervical carcinoma is amenable to prevention by population screening, as it evolves through well-defined noninvasive intraepithelial stages, which can be distinguished morphologically (4). Squamous intraepithelial abnormalities may be classified by using three-tier (CIN) or two-tier (Bethesda) systems. As classified by the Bethesda system, low-grade squamous intraepithelial lesions (LSIL), corresponding to CIN1 and cervical HPV infection, generally represent productive HPV infections with a relatively low risk of progression to invasive disease (5). High-grade squamous intraepithelial lesions (HSIL), corresponding to CIN2 and CIN3, show a higher risk of progression than LSIL though both LSIL and HSIL are viewed as representing a potential precursor of malignancy. The introduction in 1943 of the Papanicolaou (Pap) smear test (6) to identify these precursor lesions has proved to be the most successful public health measure introduced for the prevention of cancer and has proven highly effective in reducing cervical cancer mortality and morbidity rates. The Pap test samples approximately 500,000–600,000 superficial surface cells from the epithelium of the cervix (exfoliative cytology). Smear preparations are made from these samples and screened for the presence of precursor malignant (dysplastic) cells by using morphological criteria. If detected early, cervical cancer is easily treated.

However, despite the introduction of mass screening programs, the best of which have reduced mortality rates by 70%, incidence of cervical cancer in the United States has been increasing by about 3% a year since 1986 in spite of an intensification of the rate of screening (7). The failure of the Pap test to eradicate this potentially preventable disease emphasizes the limitations of this screening method. It is prone to errors at all levels, including taking the smear, identifying and interpreting abnormalities in the cytological specimen, and doing inadequate follow-up procedures (8). Consequently, high numbers of false-negative results (20–40%) are associated with this test (7). This failure partly reflects the subjectivity of cytological diagnosis and the limited time available for screening each slide because of excessive workloads. Hence abnormal cells are missed, especially if the proportion of abnormal cells in the smear is low because of inadequate sampling. In this study we have identified human Mcm5 and Cdc6 proteins as markers for cytological assessment that can improve the detection efficiency for precursor malignant cells in the Pap smear test. This detection method can be combined with Pap stain to give an immunoenhanced Pap smear test.

Established cell proliferation markers such as Ki-67 and proliferating cell nuclear antigen (PCNA) have not been useful for cervical smear analysis. We have examined two proteins involved in the regulation of DNA replication, namely Cdc6 and Mcm5. These proteins are sequentially assembled into a prereplicative complex or ''replication license'' that is essential for the initiation of DNA replication. Disassembly of this complex during replication serves as a ratchet to prevent reinitiation of replication within a single cell cycle (9–11). These highly conserved proteins appear to be essential for initiation of DNA replication in all types of eukaryotic cells tested so far (11).

We report here that antibodies against Cdc6 and Mcm5 are powerful markers of cell proliferation and that they have major diagnostic potential for detecting abnormal precursor malignant cells in cervical smears and biopsies. Immunostaining with these antibodies can be superimposed over the conventional Pap stain so that the advantages of both methods can be exploited.

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Abbreviations: HPV, human papilloma virus; SIL, squamous intraepithelial lesion; HSIL, high-grade SIL; LSIL, low-grade SIL; Pap, Papanicolaou; PCNA, proliferating cell nuclear antigen; TBS, tris-buffered saline.

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MATERIALS AND METHODS

Clinical Materials. Cervical biopsies were obtained from specimens taken at colposcopy clinics. Twenty-six biopsies showed HSILs, and 13 showed LSILs. Normal cervical tissue was obtained from age-matched patients undergoing hysterectomy for diseases unrelated to the cervix ($n = 18$). The tissue was snap-frozen in liquid nitrogen and stored at −70°C. Tissue from each specimen also was fixed in formalin and processed to paraffin. In each case, histological appearances in frozen sections corresponded to those in paraffin sections.

Cervical smears were obtained at colposcopy clinics in the East Anglian region. In each case smears and/or cervical biopsies also were obtained for routine processing and assessment in the local histopathology department. Ethical approval for collection of cervical specimens was provided by Health Authority District Ethical Committees.

Cervical smears obtained from colposcopy outpatient clinics at local hospitals were subjected to a preliminary small blinded trial. A comparison was made between the standard Pap stain and the combination of the Pap stain with immunostaining by using antibodies against Mcm5 (immunoenhanced Pap test) to compare the detection efficiency for SIL cells. The patients underwent clinical assessment by a consultant gynecologist, after which a smear was taken for assessment by Mcm5 immunostaining. In all cases an independent pathological diagnosis was made in a routine hospital pathology laboratory from an excision biopsy and/or conventionally Pap-stained smear obtained before the test smear. The test smear was assessed for the presence or absence of positively immunostained cells. Results subsequently were correlated with the diagnosis made by routine histology and/or cytology.

Production of Rabbit Polyclonal Antisera Against Human Cdc6 and Mcm5 Proteins. Polyclonal antibodies against human Mcm5 and Cdc6 proteins were raised and affinity-purified as described for XMcm7 (12). Human expressed sequence tags encoding a putative human homologue of Cdc6 were identified on the basis of their homology to yeast Cdc6/Cdc18 and human Orc1. Corresponding cDNA clones (110966, 204214, and 294716; Image Consortium, Research Genetics, Huntsville, AL) were sequenced by primer walking. Fragments corresponding to amino acids 145–360 and 364–547 were subcloned into pET23a expression vector (Novagen) and expressed as His-tag fusion proteins in *Escherichia coli* CL41 strain (13). The expressed proteins were purified by nickel affinity chromatography and used to immunize rabbits. Immunization protocol and affinity purification of antibodies were performed as described (12).

Cell Culture. Newborn human fibroblasts were grown to confluence in α-MEM (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 10 units/ml of penicillin (Sigma), and 0.1 mg/ml of streptomycin (Sigma) and kept in a confluent quiescent state for 2–7 days (G_0 arrest). The culture medium was replaced every 2 days. G_0 cells were released into the proliferative cell cycle by subculturing at 20–30% cell density in fresh medium. Entry into G_0 and release back to the proliferative cell cycle were monitored by BrdUrd incorporation (50 μM) in a 1-hr pulse.

Indirect Immunoperoxidase Staining. Serial frozen sections (5 μm) were fixed in acetone for 10 min. Endogenous peroxidase activity was quenched by incubation in 0.6% hydrogen peroxide/100% methanol for 30 min. Sections were washed in tris-buffered saline (TBS) and blocked overnight with 10% goat serum in TBS. Primary antibodies were diluted [PCNA (Novocastra) 1:100, Ki-67 (Dako) 1:20, Mcm5 1:200, and Cdc6 1:400] in TBS containing 1% BSA, and 100 μl was added to each section. Incubation was overnight at 4°C in a humidified chamber. The slides then were washed in TBS, followed by biotinylated goat anti-rabbit secondary antibody (Dako) at 1:500 in TBS containing 1% BSA for 30 min at room temperature. After washes in TBS, streptavidin horseradish peroxidase (HRP, Dako) was added. After additional washes, the presence of HRP was detected with the substrate diaminobenzidine (Sigma). The reaction was stopped by rinsing in water, and slides were lightly counterstained with hematoxylin, dehydrated through graded ethanols, and cleared in xylene. Coverslips were applied with DPX mounting medium (Gurr, BDH).

Cervical Smear Preparation. Fresh smears were fixed for 5 min in acetone/methanol (50:50). For immunostaining, endogenous peroxidase activity was quenched (as above), cells were permeabilized with 4 mM sodium deoxycholate for 10 min, washed with TBS containing 0.25% Triton X-100, and blocked overnight with 10% goat serum in TBS. Primary antibodies were diluted as above in TBS containing 1% BSA and incubated overnight at 4°C. Washing steps and the secondary antibody step were performed essentially as described above for sections. Smears were either lightly counterstained with hematoxylin (see Fig. 3) or standard Pap stain (6) (see Fig. 4) before mounting.

Immunofluorescence. Smears for immunofluorescence were fixed, quenched, permeabilized, and stained with the primary and secondary antibodies as above. Secondary antibody then was detected through an enhanced streptavidin/biotin system. The biotinylated anti-rabbit antibody was detected by streptavidin-conjugated fluorescein (Amersham), followed by biotinylated antistreptavidin (Vector), and finally streptavidin-conjugated fluorescein again. DNA was counterstained with propidium iodide/RNase A (both Sigma at 50 ng/ml). Slides were washed and mounted in 90% glycerol/PBS/phenylenediamine. Fluorescent images (see Fig. 4*D*) were collected on a Bio-Rad MRC 1024 scanning laser confocal microscope.

Appropriate controls were performed in all systems with preimmune serum, omission of antibody layers, and preabsorption of Cdc6 and Mcm5 antibodies with the relevant antigen before use. In all cases, they gave negative immunostaining.

RESULTS

Cdc6 and Mcm5 Proteins Are Down-Regulated in Quiescent Cells. We first looked for differences in expression of proteins that regulate DNA replication (origin recognition complex, Cdc6, and Mcm5 proteins; refs. 11 and 14–16) between proliferating and quiescent cells. Although the abundance of proteins of the origin recognition complex does not vary even when cells are made quiescent (data not shown), Fig. 1 shows that Cdc6 protein is absent from quiescent (G_0) newborn human fibroblasts but that it is detectable once cells enter the proliferative cell cycle. Down-regulation of Cdc6 also has been

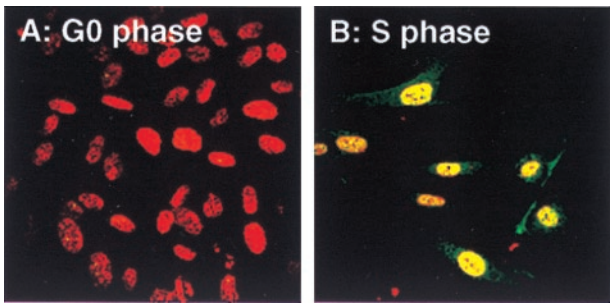

FIG. 1. Cdc6 is expressed in cycling but not quiescent human cells. (*A*) Contact inhibited, quiescent, or (*B*) synchronized S phase newborn human fibroblasts were grown on coverslips and stained with propidium iodide to reveal DNA (red) and with anti-Cdc6 antibody followed by secondary fluorescein isothiocyanate-conjugated antibody (green: note that green + red = yellow). Magnification: ×130.

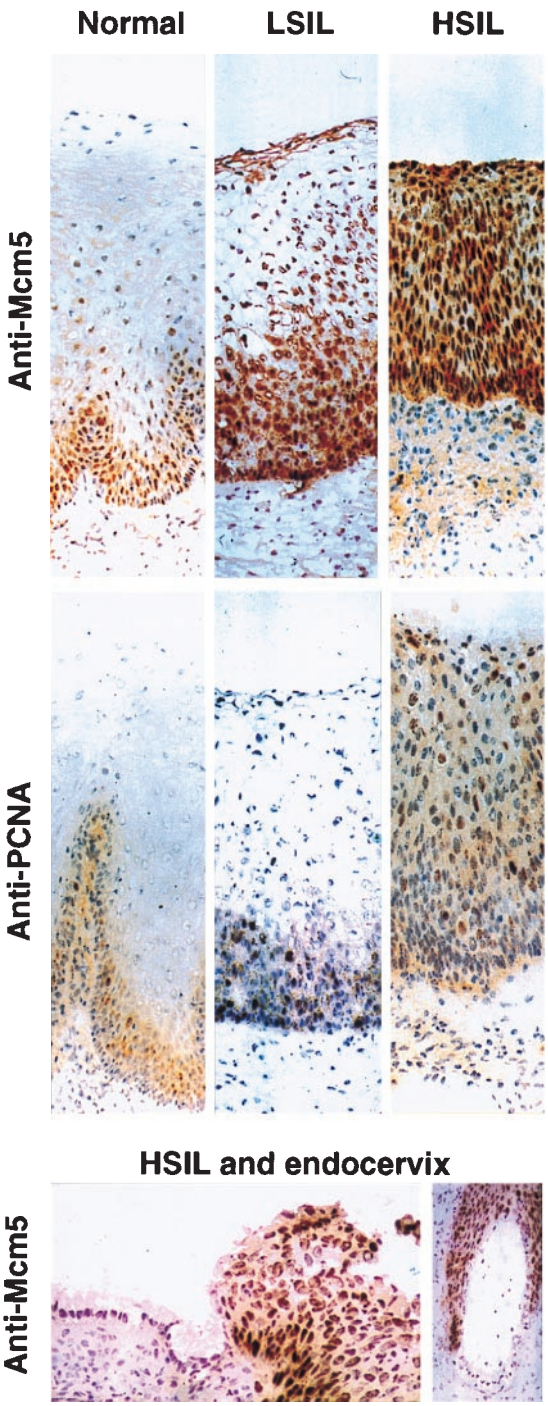

FIG. 2. Immunoperoxidase staining of frozen sections of normal cervix, LSILs, and HSILs with antibodies against PCNA and Mcm5. Frozen sections of normal cervix, LSILs, and HSILs were immunostained for Mcm5 or PCNA (as a conventional proliferation marker) by the immunoperoxidase method. In normal cervix, surface cells likely to be sampled by cervical smear examination are negative with both antibodies. In LSIL with associated koilocytosis (cells showing HPV cytopathic effect), anti-PCNA antibodies stain basal and some parabasal nuclei, but surface koilocytes are negative. In contrast, anti-Mcm5 antibodies stain nuclei in superficial as well as basal epithelial layers including most koilocytes. In HSIL, anti-PCNA antibodies show focal staining of 10% of nuclei in the surface layers, whereas anti-Mcm5 antibodies stain virtually all nuclei, including those at the epithelial surface. Antibodies against Ki-67 (another conventional proliferation marker), show essentially similar results to anti-PCNA. In contrast, antibodies against Cdc6 stain all cells in HSIL as seen with anti-Mcm5 (data not shown; *Top* and *Middle*; magnification ×108). Although HSIL cells show strong nuclear immunostaining with anti-Mcm5, endocervical surface, and glandular cells are negative (*Bottom Left,* magnification ×225; *Bottom Right,* magnification ×87).

observed in quiescent Wi38 fibroblasts (14). Similarly, Mcm5 is absent from the chromatin-bound fraction of quiescent cells and lost from cells gradually during quiescence (data not shown). Identical results were obtained for murine NIH 3T3 cells (K.S., A.D.M., Y. Kubota, T. Krude, P.R., K. Marheineke, R.A.L., and G.H.W., unpublished work).

Antibodies Against Mcm5 and Cdc6 Specifically Detect Abnormal Precursor Malignant Cells in Cervical Tissue Sections. These studies suggest that anti-Cdc6 and anti-Mcm5 antibodies, but not anti-origin recognition complex antibodies, might provide specific markers for cells in the proliferative cycle. To determine whether these proliferation markers can be used to detect abnormal precursor malignant cells in cervical smears we tested affinity-purified polyclonal antibodies raised against human Cdc6 and Mcm5 proteins on serial sections of normal and abnormal human uterine cervix and on cervical smears. Immunostaining with these antibodies was compared with the staining pattern obtained with conventional proliferation markers PCNA and Ki-67. Although all were expected to perform as proliferation markers, striking differences were observed in their potential diagnostic value. The staining pattern of frozen tissue sections of normal cervix was similar with all four antibodies (Fig. 2 and data not shown). All showed staining of the basal proliferating layers of the cervical squamous epithelium but did not stain the superficial differentiating cells. However, marked differences were observed between antibodies when applied to frozen tissue sections of both HSIL and LSIL (Fig. 2 and data not shown). Specifically, antibodies against Cdc6 and Mcm5 were found to stain a much higher proportion of abnormal cells than antibodies against Ki-67 or PCNA in both grades of lesion (Table 1). Although full thickness staining of the epithelium was observed with all antibodies for HSILs, only antibodies against Cdc6 and Mcm5 stained both the basal and superficial layers in LSILs. Interestingly, koilocytes, cells that show HPV cytopathic effect, stained strongly for Cdc6 and Mcm5 but not for PCNA or Ki-67 (Fig. 2). This high degree of sensitivity using antibodies against Mcm5 and Cdc6 was accompanied by a high degree of specificity; adjacent normal superficial ectocervical, metaplastic, stromal, and inflammatory cells were not stained. Negative immunostaining with anti-Mcm5 and anti-Cdc6 antibodies also was observed for surface and glandular endocervical epithelium (Fig. 2, *Bottom*). The specificity of immunostaining with anti-Mcm5 or anti-Cdc6 antibodies was confirmed by blocking with the bacterially expressed proteins used to raise the antibodies (data not shown). Identical patterns of immunostaining were observed within each category during examination of 57 biopsies (26 HSIL, 13 LSIL, and 18 normal), indicating that antibodies against Mcm5 or Cdc6 are of major potential diagnostic value for detecting abnormal precursor malignant cells.

Antibodies Against Mcm5 and Cdc6 Specifically Detect Abnormal Precursor Malignant Cells in Cervical Smears. Cervical smear tests sample cells from superficial surface layers of the cervical epithelium. These surface layers normally should contain only nonproliferating differentiated cells. Results with cervical tissue specimens suggest that anti-Mcm5 and anti-Cdc6 antibodies may be important for diagnosis in the cervical screening test (Table 1). Cells obtained by cervical smear examination from normal, metaplastic, and squamous intraepithelial lesions were examined with all four antibodies. Normal ectocervical, endocervical, metaplastic, and inflammatory cells showed no immunostaining with any of the antibodies tested (Fig. 3). However, as in the case of the cervical tissue sections, marked differences were seen in the immunostaining of abnormal cells derived from LSILs or

Table 1. Percentage of immunostained cells in the most superficial five layers in sections of HSIL and LSIL cervix similar to Fig. 2

	HSIL				LSIL			
	Mcm5	Cdc6	PCNA	Ki-67	Mcm5	Cdc6	PCNA	Ki-67
Median*	95	90	10†	5‡	53	43	3§	1¶
Range	80–100	78–100	5–15	3–8	10–70	8–65	0–20	0–10

*Each value is calculated from five different sections. Wilcoxon Rank Sum Test, P values are two-sided:
†PCNA vs Mcm5 $P < 0.01$; PCNA vs Cdc6 $P < 0.01$;
‡Ki-67 vs Mcm5 $P < 0.01$; Ki-67 vs Cdc6 $P < 0.01$;
§PCNA vs Mcm5 $P < 0.05$; PCNA vs Cdc6 $P < 0.05$;
¶Ki-67 vs Mcm5 $P < 0.05$; Ki-67 vs Cdc6 $P < 0.05$.

HSILs. Although Ki-67 and PCNA showed nuclear staining of only a minority population of the abnormal precursor malignant cells, Mcm5 and Cdc6 showed strong immunostaining of the majority of the abnormal cells in the smear (Fig. 3). High specificity and sensitivity were observed during examination of 92 smears. This high degree of sensitivity was observed for all subtypes of SILs, including immature (basaloid type), mature (keratinizing), and metaplastic as well as for abnormal cells in three cases of glandular neoplasia/adenocarcinoma. Thus the sensitivity and specificity of antibodies against Cdc6 and Mcm5 for detection of abnormal precursor malignant cells in frozen sections also is observed in cervical smear preparations.

To improve the detection efficiency of abnormal cells in the standard Pap test we have combined Mcm5 and Cdc6 immunostaining with the Pap stain (immunoenhanced Pap test). This combined approach means that the additional information from immunostaining can be added without loss of any morphological information from the Pap stain (Fig. 4 *A* and *B*). At high magnification it is still possible to undertake detailed examination of cellular and nuclear morphology even with superimposition of the immunoperoxidase stain (Fig. 4*C*), which should greatly facilitate introduction of the improved method into the clinic.

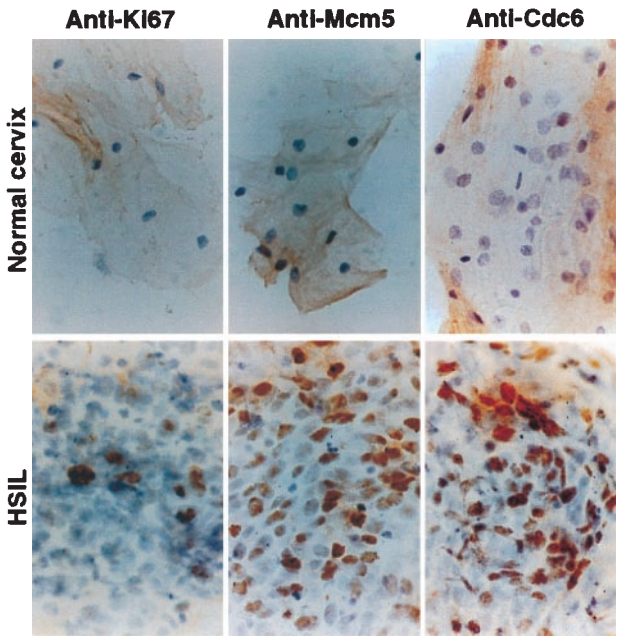

FIG. 3. Immunoperoxidase staining of cells obtained by cervical smear examination from surface of normal ectocervix or HSIL with antibodies against Ki-67, Mcm5, or Cdc6. Superficial squamous cells from normal ectocervix show no nuclear staining with any of the antibodies tested. In HSIL, anti-Ki-67 antibodies (and anti-PCNA; data not shown) show nuclear staining of only a minority population of the abnormal cells in the exfoliated sheets. In contrast, anti-Mcm5 and anti-Cdc6 antibodies stain most HSIL cells. Magnification: ×240.

An Immunoenhanced Pap Test Shows Increased Efficiency of Detection of Abnormal Cells in the Cervical Smear Test. A small blinded trial was performed to compare the immunoenhanced Pap test with the standard Pap test for detection efficiency of abnormal cells. Of the 58 cases examined in the trial, the routine pathological diagnosis was made by histology alone in six cases, by cytology alone in 34 cases, and by histology and cytology in 18 cases. Table 2 shows that of 28 cases assessed as positive for LSIL, HSIL, or endocervical adenocarcinoma, in 22 of which a histological diagnosis was made, all 28 also were classified as positive by our immunoenhanced Pap test. In one case of HSIL diagnosed by biopsy, a smear also was taken for routine Pap assessment. This smear was reported as negative, whereas abnormal cells were detected by immunostaining for Mcm5. Of 30 cases classified as normal by routine Pap stain, 26 also were identified as negative by our immunoenhanced Pap test. Of the remaining four cases classified as normal by routine Pap stain but as positive by our immunoenhanced Pap test, two cases contained immunostained immature metaplastic squamous cells showing reactive changes in an inflammatory background. These were readily classified in the immunoenhanced Pap test using the underlying Pap stain. The other two cases were confirmed as containing abnormal (LSIL) cells on re-examination of the Pap stain by the local pathologist; i.e., they were false-negatives of the type we are attempting to eliminate. On subsequent excision biopsy each of these two latter cases actually were found to contain areas of HSIL on histological examination, yet they had been reported as negative by conventional Pap stain.

The striking sensitivity and specificity of antibodies against these proteins for identifying abnormal cells in cervical smears raises the possibility of automating the cervical screening test. An indirect immunofluorescence detection approach, a method that might facilitate automation, therefore was applied to cervical smears, and again striking specificity was observed with both the Mcm5 and Cdc6 antibodies (Fig. 4*D* and data not shown).

DISCUSSION

The Pap smear test (6) has been extremely effective in reducing cervical cancer rates. However, this complex test is prone to errors at multiple levels, resulting in a high false-negative rate of 20–40% (7). A major factor contributing to the false-negative rate is the failure of detection of abnormal precursor malignant cells (8). This failure is most likely to occur in routine screening where there are a low number of abnormal cells caused by sampling error combined with insufficient screening time as a result of excessive workloads. Here we demonstrate that antibodies against recently discovered DNA replication regulatory proteins can facilitate the detection of abnormal cells and help to reduce the high false-negative rate.

Many oncogenes encode growth factors, receptors, or proteins of signal transduction pathways (17). These pathways are inherently redundant, so that no single oncogene can be a

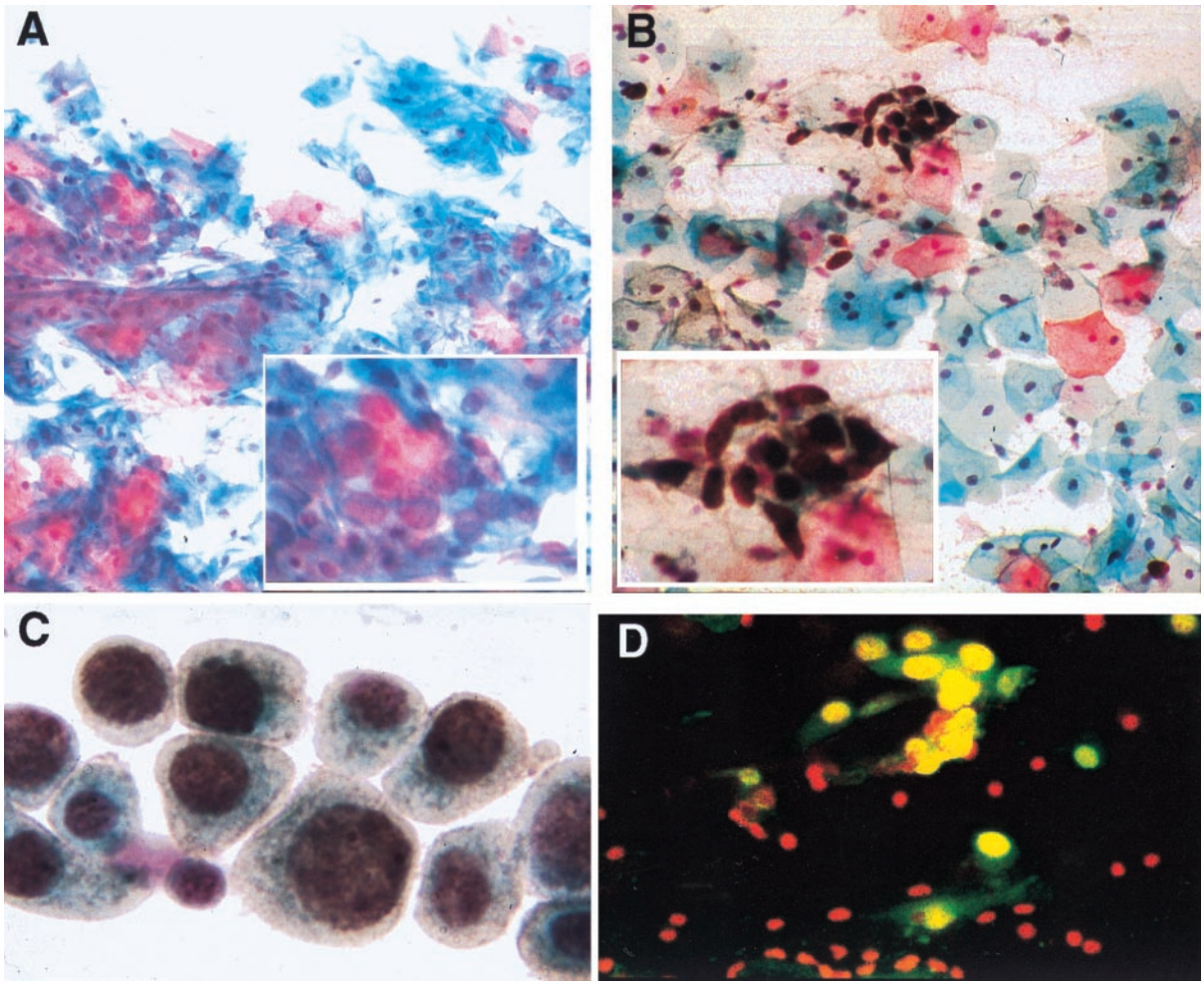

FIG. 4. Staining of HSIL cells in cervical smears by Pap stain alone (*A*) and with anti-Mcm5 antibody using a combination of immunoperoxidase and Pap staining (*B* and *C*) or immunofluorescence (*D*). (*A* and *B*) Cells are viewed at the scanning magnification used in the standard cervical screening test. The field in *A* stained with Pap stain alone contains several foci of HSIL cells (one is shown in detail, *Inset*), although they are difficult to recognize. The field in *B* stained with Pap stain and anti-Mcm5 antibodies contains immature-type HSIL cells, which are readily identified by the anti-Mcm5 antibody. As the Pap stain also has been performed on this latter sample, no information derived from the Pap stain has been lost (magnification: ×130). The field in *C* contains metaplastic type HSIL cells. Superimposition of the immunoperoxidase method on the Pap smear still allows detailed examination of the cellular and nuclear morphology of positively immunostained cells. The irregular nuclear outlines, prominent nucleoli, and abnormal chromatin patterns are still clearly visible (magnification: ×400). (*D*) Predominant nuclear immunofluorescence of Mcm5 (green), with a low level of cytoplasmic staining. DNA is counterstained with propidium iodide (red) so that immunoreactive nuclei appear yellow. HSIL cells stain positively, whereas the surrounding squamous and metaplastic cells are negative (magnification: ×243).

reliable marker for all neoplastic cells. However, signaling pathways converge at the point of initiation of DNA replication. Minichromosome maintenance proteins (Mcm) and Cdc6 are essential for the key regulatory step of initiation of DNA replication in all eukaryotes investigated so far (9–11, 18, 19).

Table 2. Comparison of the immunoenhanced Pap test with the conventional pathological diagnosis

Classification by Anti-Mcm5 test	Classification by conventional criteria			
	Normal	LSIL	HSIL	Adeno-Carc.
Negative	26	0	0	0
Positive	4*,†	9	16‡	3
Total	30	9	16	3

*Two smears that were reported normal by the conventional Pap test stained positively by anti-Mcm5 antibody. On this basis the patients were recalled and were shown histologically (approximately 6 months after the original smear) to have HSIL.

†Two antibody-positive smears were found to contain just immature metaplastic squamous cells, which were recognized by conventional criteria using the underlying Pap stain on the same slide.

‡Of these 16 cases, 12 were confirmed by both Pap smear and histology.

Observations have shown that these proteins are present throughout the cell cycle of proliferating cells, but not in nonproliferating quiescent cells (Fig. 1; ref. 14), and our data demonstrates that absence of these proteins on chromatin is a cause of quiescence, rather than a consequence (K.S., A.D.M., Y. Kubota, T. Krude, P.R., K. Marheineke, R.A.L., and G.H.W., unpublished work). We have tested antibodies against Mcm5 and Cdc6 to distinguish abnormal proliferating cells from a background of normal, nonproliferating differentiated cells in cervical smears.

Our data obtained from 92 smears and 57 biopsies show that abnormal precursor malignant cells of SILs (dysplastic cells) can be detected with high specificity and sensitivity by either immunoperoxidase (Figs. 2, 3, and 4 *A–C*) or immunofluorescence staining (Fig. 4*D*) using antibodies against Cdc6 or Mcm5. One of the reasons these antibodies appear to be superior for diagnosis compared with antibodies against PCNA and Ki-67 relates to the preservation of these antigens in cytological material. Although similar results have been obtained by other investigators and ourselves when comparing antibodies against PCNA with either anti-Mcm5 or anti-Mcm7 antibodies on formalin-fixed, paraffin wax-embedded tissue

sections (data not shown; refs. 20 and 21), we show here that the sensitivity for Mcm5 and Cdc6 is much higher than for PCNA or Ki-67 when applied to cervical smears and frozen sections.

To date dysplastic cells have been characterized by the classical morphological features of maturation arrest and associated cytological abnormalities. We suggest that dysplastic cells can alternatively be recognized by their persistence in the cell cycle compared with normal epithelial cells that exit the cell cycle during maturation and differentiation. We have exploited this difference by using antibodies directed against proteins of the prereplicative complex, which are characteristic of cycling, but not quiescent cells, to provide a powerful complementary test that can be combined with the standard Pap stain test. Our results show that this immunoenhanced Pap test has the potential to increase the detection efficiency for precursor malignant cells in the cervical smear test for the most clinically significant abnormalities. In a small preliminary blinded trial of 58 patients we successfully have detected abnormal immunostained cells in all 28 cases that were independently assessed as abnormal by standard Pap stain in routine hospital laboratories. Importantly, two additional immunostained cases initially were recorded as negative by routine Pap stain. However, on review those two cases were later reclassified as LSIL. Follow-up cervical biopsies showed that those two patients were actually HSIL on histological examination. Those two latter examples illustrate the potential of the antibodies to decrease false-negative cases. Further studies will be required to determine the value of the antibodies in identifying other, less well-defined abnormalities such as AGUS (atypical glandular cells of uncertain significance) or ASCUS (atypical squamous cells of uncertain significance), but the Pap stain of the same smears still can contribute to these classifications.

It is possible that some of the more unstable and immature phases of squamous metaplasia may be detected in the immunoenhanced test because these cells are immature and may still be in cell cycle (Table 2). However, these cells are identified only in a minority population of cervical smears, and they can be readily identified by morphological criteria using the Pap stain.

In summary we have developed a modification of the standard Pap cervical smear test in which we have introduced an immunostain for detection of abnormal precursor malignant cells. The immunoenhanced Pap test identifies abnormal cells both in terms of morphological and functional criteria, the latter by using antibodies against proteins of the prereplicative complex, characteristic of cycling cells. This complementary approach should markedly improve the accuracy, precision, and sensitivity of the cervical smear test. Furthermore the immunoenhanced Pap test is a method that has the potential for automation. Large-scale clinical trials will be required to fully evaluate this approach, and these trials will require mAbs for full reproducibility and standardization.

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