CLINICAL RESEARCH

Human papillomavirus infection of the uterine cervix of women without cytological signs of neoplasia

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Abstract

One hundred and six patients were studied whose cervical smears showed only non-specific inflammatory changes. Screening for genital pathogens yielded only a few positive cases. Histological examination of biopsy specimens taken by colposcopically directed tissue sampling showed cervical intraepithelial neoplasia in 13 of the women (12·3%). Deoxyribonucleic acid (DNA) hybridisation techniques were used to detect human papillomavirus, which was found in 24 patients (22·6%).

In a second group of 104 patients with normal cervical cytology tissue biopsy samples were obtained and examined histologically but in no case was cervical intraepithelial neoplasia found. On DNA hybridisation, however, 12 patients (11·5%) were found to be positive for human papillomavirus. In this group finding human papillomavirus DNA was usually associated with a columnar ectopy.

An association between human papillomavirus type 16 DNA and both cervical intraepithelial neoplasia and cervical cancer is well established. In this study it was type 16 which occurred most frequently in both groups.

Introduction

Consequent on the colposcopic identification of cervical condylomas, Meisels *et al* in 1977 postulated that wart virus infection of the cervix might be a precursor of cervical neoplasia.[1] This hypothesis has been reinforced both by histological evidence of viral infection associated with cervical intraepithelial neoplasia and cervical cancer[2] and by DNA-DNA (deoxyribonucleic acid) hybridisation using specific human papillomavirus DNA probes.[3-7] Indeed, human papillomavirus types 16 and 18 were both originally identified from cervical carcinoma tissue.[8,9]

This study was initiated because of concern that a small but appreciable number of women, especially in the under 35 year age group, whose previous cervical smears showed only inflammatory changes without the intermediate changes of cervical intraepithelial neoplasia were presenting with invasive cervical carcinoma (R Hunter, personal communication). The study was designed to identify, using bacteriological, virological, and chlamydial culture and colposcopy, histology, and molecular biology, any potential causative agent(s) of these inflammatory cervical smears. For the purpose of the study a non-specific inflammatory cervical smear was defined as one showing polymorphonuclear leucocytes and degeneration (intracytoplasmic vacuoles, perinuclear halo formation, and pyknosis)[10] in the absence of any histological evidence of specific infective agents.

Patients and methods

The study was approved by the district ethical committee and the informed consent of each patient obtained. The study group consisted of women with non-specific inflammatory cervical smears who were invited to attend a colposcopy clinic for further investigation. The age range of the group was 16-69 (mean 29·1) years.

A second group of women with normal cervical smears who were receiving surgical treatment in hospital for conditions not associated with abnormal cytology—for example, hysterectomy for menorrhagia—were studied in parallel with the main group. The age range of this group was 17-70 years (mean 36·9). Biopsy specimens from the transformation zone were obtained at the time of surgery. All patients had been referred to the gynaecology clinic by their general practitioners for various gynaecological symptoms not associated with cervical disease.

INVESTIGATIONS

At the colposcopy clinic a standard "charcoal" cervical swab was taken for microbiological culture and placed in Amies's transport medium. "Plain" cotton wool cervical swabs were taken for chlamydial and virological culture

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and placed in the appropriate transport medium. All of these specimens were taken directly to the appropriate hospital laboratories. Routine cultures were set up to detect *Neisseria gonorrhoeae*, *Candida albicans*, and aerobic and anaerobic bacteria. *Trichomonas vaginalis* was identified by a fixed stain method with acridine orange. The viral swab was inoculated on to specific cell lines—for example, HEp_2 and vero—a cytopathic effect indicating a viral infection. The chlamydial swab was inoculated on to cyclohexamide treated McCoy cells. These cultures were then stained with polyclonal antichlamydial rabbit serum, which reacts with all trachomatis subtypes.

Colposcopy of the cervix was performed and any areas of atypia identified. Wherever possible two adjacent biopsy specimens were taken from the identified lesions by colposcopically directed tissue sampling. If no lesion was visible then two specimens were taken adjacently but at random from the transformation zone, including the squamocolumnar junction when possible. One specimen was fixed in formalin for routine histological examination and the other snap frozen in liquid nitrogen for future DNA hybridisation.

In the second group random biopsy specimens for DNA hybridisation were taken from the transformation zone at the time of surgery. The remainder of the specimen was fixed in formalin for routine histological examination.

Molecularly cloned human papillomavirus DNA—Plasmids containing cloned DNA of human papillomavirus types 1,[11] 2,[12] 4,[11] 6,[13] 10,[14] 11,[15] 16,[8] and 18[9] were propagated in *Escherichia coli* and their DNA prepared by established methods.[16]

Preparation of cellular DNA, restriction endonuclease digestion, gel electrophoresis, and blotting—The frozen tissue samples were cut into small pieces and disrupted in a microdismembranator (F-T Scientific Instruments, Gloucester).[17] Cell nuclei were prepared by the method of Favaloro *et al*[18] and the nucleic acids extracted from the nuclear pellet as described. The DNA was precipitated with ethanol, washed with 70% ethanol, dried, and resuspended in 10mM trometamol (TRIS) and hydrochloric acid pH 7·9 and 1mM edetic acid. The DNA concentration was measured by the mithramycin assay.[19] Samples of cellular DNA (10 μg) were digested with restriction endonuclease *Pst* I or *Bam* HI and fractionated on 0·7% agarose gels, as described.[20] Samples of DNA from a known human papillomavirus negative normal cervix and a human papillomavirus positive cervical carcinoma or condyloma acuminatum were included on each gel as negative and positive controls. *Pst* I or *Bam* HI cleaved, molecularly cloned DNA of human papillomavirus types 6, 11, 16, and 18 served as markers. After electrophoresis the gels were washed twice for five minutes in 0·25M hydrochloric acid[21] and the DNA transferred to nitrocellulose[22] (Schleicher and Schuell) or Hybond (Amersham) membranes. The membranes were then baked at 80°C in a vacuum oven.

DNA-DNA hybridisation—A mixed probe containing cloned DNA from human papillomavirus types 1, 2, 4, 6, 10, 11, 16, and 18 was labelled with phosphorus-32 by nick translation[23] and hybridised to the filters as described by Johnson *et al*.[24] The filters were finally washed twice for 30 minutes in 0·6 SSC (1 SSC is 0·15M sodium chloride, 0·015M trisodium citrate) and 0·1% sodium dodecyl sulphate at 41°C (low stringency, Tm −40°) or 0·6 SSC and 0·1% sodium dodecyl sulphate at 60°C (high stringency, Tm −20°), as described.[25] They were then dried and autoradiographed for one to seven days using preflashed Fuji RX *x* ray film. The autoradiographs were developed using an Agfa-Gevamatic 60 processor.

TABLE II—*Findings in 106 patients with non-specific inflammatory cervical smears and 104 patients with negative cytology*

	Normal	Inflammatory	Warty (viral)*	Cervical intraepithelial neoplasia/atypia*
	Patients with non-specific inflammatory cervical smears			
Histology	36	43	14	13
Colposcopy	38	1	9†	64
	Patients with negative cytology			
Histology‡	57	25	0	0

*Criteria for these changes based on well recognised features (summarised in ref 28).
†Six of these patients included in cervical intraepithelial neoplasia/atypia group.
‡Histological study not performed in 22 patients of group.

TABLE I—*Microbiological findings in 106 patients with non-specific inflammatory cervical smears*

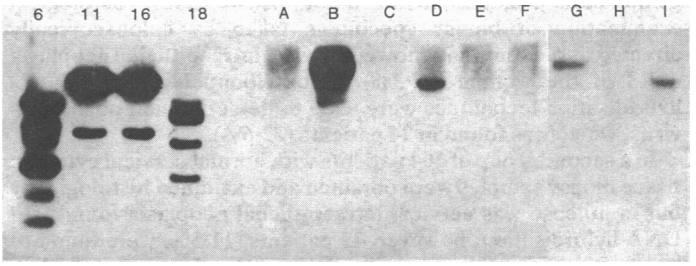
	Bacteria	*Trichomonas vaginalis*	*Candida albicans*	Herpes simplex virus	Cytomegalovirus	*Chlamydia trachomatis*
No of positive isolations	6*	0	2	0	1	2

*Five of these isolations were β haemolytic streptococcus, which was considered to be a normal vaginal commensal.

Results

Microbiology—Cultures for pathogenic bacteria, viruses, and chlamydia resulted in only a few positive isolations (table I).

Histology and colposcopy—Table II summarises the colposcopic findings in the group with non-specific inflammatory cervical smears and the histological findings for both groups of patients. Though many cervices appeared to show colposcopic atypia, cervical intraepithelial neoplasia was not found histologically in all cases, but many of these atypical areas contained inflammatory changes. This may be explained by the fact that the cervices were examined extremely critically colposcopically for the slightest evidence of any acetowhite area. Acetowhite areas which are considered to be normal such as immature squamous metaplasia, regenerating squamous epithelium, and the "congenital transformation zone" would therefore have been included in the classification. The group of patients with negative cervical cytology showed no evidence of cervical intraepithelial neoplasia or viral infection, but 25 of the 104 patients showed inflammatory changes.



Low stringency blot hybridisation (Tm −40°) of DNA from cervical biopsy specimens using ^{32}P labelled mixed human papillomavirus DNA probe. DNA samples digested with restriction enzyme *Bam* HI and products fractionated on 0·7% agarose gel. Lanes 6, 11, 16, and 18 are marker tracks of respective molecularly cloned human papillomavirus DNA cleaved from plasmid vector with appropriate restriction enzyme. DNA obtained from normal cervix (lane A), squamous cervical cancers (lanes B and G), and patients with non-specific inflammatory cervical smears (lanes C, D, E, F, H, and I). Specific single probe DNA hybridisation followed by high stringency wash (Tm −20°) used to determine that human papillomavirus DNA in lanes D and I was type 16. Lanes C, D, and I showed following changes respectively: cervical intraepithelial neoplasia stage III, cervical intraepithelial neoplasia stage II, and koilocytosis; remainder showed inflammatory changes. Human papillomavirus type 16 DNA in squamous cell cancers (lanes B and G) appeared to be of larger molecular weight than viral unit length, suggesting that viral DNA was integrated into host cell DNA.[42,43] In non-specific inflammatory cervical smears and normal samples human papillomavirus DNA appeared to be unit length, presumably episomal forms.

DNA-DNA hybridisation—The figure shows examples of Southern blot hybridisation analyses. Table III shows that human papillomavirus type 16 was the most frequently found in both groups of women. Human papillomavirus DNA was found in 24 of the 106 patients with non-specific inflammatory cervical smears, roughly half of them coming from the category histologically positive for cervical intraepithelial neoplasia or virus. The remainder showed a mainly inflammatory or, less often, normal histological picture, which was usually associated with a "columnar ectopy." Of the 104 patients with negative cytology, 12 were positive for human papillomavirus DNA. Half of these came from the group with histological

TABLE III—*Summary of prevalence of human papillomavirus DNA*

	Total No	Human papillomavirus					Positive		
		Total	6	11	16	18	Mixed	Others*	Negative
Non-specific inflammatory cervical smears	106	24	—	2	16	—	1	7	82
Non-specific inflammatory cervical smears with ectopy	28	7	—	—	5	—	—	2	21
Non-specific inflammatory cervical smears positive histologically for cervical intraepithelial neoplasia or virus	27	12	—	2	8	—	1	3	15
Non-specific inflammatory cervical smears appearing abnormal colposcopically	70	16	—	2	10	—	1	5	54
Normal smears	104	12	—	—	9	—	—	3	92
Normal smears with ectopy	19	7	—	—	5	—	—	2	12

*Samples positive for human papillomavirus DNA at low stringency using mixed probe but negative at high stringency with specific probes listed.

evidence of inflammatory changes. Seven of the 12 patients positive for human papillomavirus DNA were found to have columnar ectopy ("cervical erosion"), and overall 14 of all 49 cervical erosions in the two groups were positive for human papillomavirus DNA.

Discussion

These results show that there was a 12·3% false negative rate for non-specific inflammatory cervical smears which were subsequently found to be cervical intraepithelial neoplasia and that a further 13·2% of these patients showed histological evidence of infection with human papillomavirus.[10] This false negative rate may have been due to several causes, which have been reviewed.[26] Probably the most important factor in this study was suboptimal cytology. On colposcopic examination many of the areas of cervical intraepithelial neoplasia were noted to be very small and may well have been missed by even an experienced sampler. No evidence of cervical intraepithelial neoplasia or viral changes was found in the samples that were histologically normal.

Microbiological culture of pathogens from these patients yielded positive results in only a few. Hence it seems unlikely that these organisms play any part in the aetiology of non-specific inflammatory cervical smears. Nevertheless, neither bacteria,[27] candida,[27] trichomonas,[28] nor chlamydia[26] are regarded as strong contenders for aetiological agents in cervical cancer. There remained a proportion of patients, however, in whom inflammatory changes were detected histologically but in whom no causative agent could be found on the basis of available techniques.

Cytomegalovirus and herpes simplex virus were found in very few samples. In the case of herpes simplex virus this was probably due to latency of the virus. Positive cultures can be obtained only during active infection. Our figures agree with previous data for cytomegalovirus[29] and herpes simplex virus.[27] Work is in progress to screen for herpes simplex virus DNA in the cervical samples by rehybridising the DNA on the filters to DNA fragments of herpes simplex virus type 2.

The incidence of chlamydial infection was lower than expected but is known to range from 28% in women attending sexually transmitted disease clinics[30] (S J Richmond, personal communication) to 11% for a control population,[29] 7% for pregnant women routinely screened in an antenatal clinic,[30] and 2·5% for all gynaecological outpatients (S J Richmond, personal communication).

From previous data DNA of human papillomavirus type 16 appears to be a risk factor for cervical cancer.[3 5-9] In this study cervical biopsy specimens from 16 of 106 patients with non-specific inflammatory cervical smears were positive for human papillomavirus type 16 DNA. This correlates with the results of Prakash *et al*, who found that 12% of patients with histologically confirmed cervicitis were positive for human papillomavirus type 16 DNA.[5] Half of the lesions of cervical intraepithelial neoplasia were positive for human papillomavirus DNA, which agrees with the findings of others.[5 7] Nevertheless, a much higher proportion might have been expected.[31 32] This discrepancy may be explained by the small size of the lesions, which made it difficult to obtain representative samples for both histological and hybridisation examinations.

In contrast with other studies[3 7] human papillomavirus DNA types 6 and 11 were found rarely or not at all (table III). Human papillomavirus type 18, though found in other countries,[9 30] was not identified in any sample. These findings agree with those of D J McCance (personal communication); human papillomavirus type 18 has, however, recently been identified in Scotland (M S Campo, personal communication).

An interesting finding was that 14 of all 49 cervices with columnar ectopy (29%) were positive for human papillomavirus DNA.[10 33] Just over half of these patients had normal cervical smears. Possibly human papillomavirus is harboured in cervical "erosions" close to the squamocolumnar junction after venereal transmission in sexually active women at risk.[34] This site is actively involved in the transformation zone which undergoes squamous metaplasia, which may facilitate entry of human papillomavirus into the cell and then initiate the process of cervical intraepithelial neoplasia, possibly being triggered by some other agent.[35] This evidence concurs with epidemiological evidence that adolescent coitus[36] and childbearing[37] increase the risk of cervical cancer. It is at these times that an erosion is likely to be present because of an excess oestrogen effect,[38] so presenting a suitable initial habitat for human papillomavirus. This hypothesis may incriminate the combined oral contraceptive pill as a risk factor because it has an excess oestrogen effect which promotes the development of columnar ectopy.[33 39] This supports the findings of Vessey *et al*, who found that long term use of the pill increased the risk of cervical neoplasia when compared with the intrauterine device as a method of contraception.[40] Our own figures (not given) showed that ectopic states were more common in those who had taken the pill recently. All of the human papillomavirus DNA positive patients with ectopy and most of the patients with non-specific inflammatory cervical smears who were positive for human papillomavirus DNA had also taken the pill recently. By comparison, most of the patients with non-specific inflammatory cervical smears who had no evidence of any abnormality had never taken or not taken the pill for some time. There appeared to be no relation of any of these abnormalities to age or parity in our series.

In this study a high proportion of human papillomavirus type 16 DNA was found. It seems likely that certain patients with columnar ectopy and non-specific inflammatory cervical smears are "at risk" groups because they have a greater chance of harbouring human papillomavirus DNA. The latter group contained an appreciable proportion found to have cervical intraepithelial neoplasia. We recommend that this group should have colposcopy, which will help to identify those whose cervices contain human papillomavirus sequences. These patients will then require close follow up or treatment, or both, as indicated by the colposcopic findings. Furthermore, it appears prudent to treat cervical ectopic states by locally destructive methods in women at risk in order to break this possible chain of infection. These methods have been found by Vonka *et al* to protect against the development of cervical neoplasia.[41]

We conclude that patients with non-specific inflammatory cervical smears should be paid as much attention as those whose cervical smears show cervical intraepithelial neoplasia. They should have frequent, regular, repeated cervical smears as a bare minimum and ideally a colposcopic examination and should not merely be ignored and said to be normal because the cytology report does not show a severe abnormality.

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100 YEARS AGO

The continental tourist is apt to be somewhat embarrassed by questions of dietary, and more especially by not unfounded suspicions regarding his water-supply. In Switzerland, the water is for the most part above reproach; but it is otherwise in many other parts of the Continent. The Englishman is almost morbidly sensitive on this point; and, when his fears are once excited, he shuns his natural beverage with perhaps undue alarm, and takes refuge in unlimited indulgence in wines and mineral waters. Here, as often, we shun Scylla to fall into Charybdis. It is well to avoid the typhoid germs possibly lurking in the water-bottle; but, to court dyspepsia by an unaccustomed indulgence in acid wines, or waters largely impregnated with mineral matter, is not well. A little claret or hock can do no harm, and, where the ordinary water-supply lies under any just suspicion, the tourist may with advantage use Apollinaris, St. Galmier, or other natural water, in which the proportion of saline material is small. The waters which contain a large percentage of carbonates, chlorides, or sulphates, are quite unsuitable for common use, especially with food. (*British Medical Journal* 1886;ii:465.)

Near the bottom of the Broad Walk in Kensington Gardens, and about a hundred yards below the Round Pond, is situated a sunken well bearing the inscription "St. Govor's Well, 1856." A few years ago, the water, which issued in a considerable stream from a leaden spout, was credited with medicinal virtues, and was distributed to children and curious people in many coloured glasses by an old woman who attended daily. We have heard of the beneficial action of the waters on crippled joints. Since the woman disappeared (from over-indulgence in the waters?) a metal cup has been attached to the spout, and the well has been the constant resort of thirsty children and nursery-maids. During the past summer, while the Round Pond has been drained St. Govor's Well has run dry, and it is said that some pipes were discovered under the two or three feet of stinking mud at the bottom of the pond which had the appearance of communicating directly with the well, so that it seems probable that the water has been filtered through the mud, and not through the intervening bed of gravel. Can its reputed medicinal virtues be due to the organic impurities of stagnant water? It is to be hoped that the source of the water will be fully investigated, and, if it is found to be the mere drainage of the pond, that the well will be closed, or else that a supply of pure water will be laid on from a known source. The well would be very much missed by the children, as there is no other drinking-fountain in the gardens. (*British Medical Journal* 1886;ii:936.)

A correspondent writes: The extended consumption of one or the other of this class of substances points to the existence of some beneficial effect to be derived therefrom, although what this consisted in it has been difficult to say, judging otherwise than subjectively. Sir William Roberts, of Manchester, has lately suggested an ingenious hypothesis, which offers a plausible explanation of their use. Man, in a state of nature, would derive his sustenance presumably from materials which, from their being raw, or at any rate imperfectly cooked, would be necessarily but slowly digested and assimilated. With civilised communities, on the contrary, everything is done with the view of faciliating digestion, by the removal of indigestible parts of the food, or by submitting them to processes which favour the action of the juices with which they are to be brought into contact. Under these circumstances, it is quite possible that digestion and assimilation may proceed at a speed not only unnecessary, but even disturbing, to the equilibrium of the organism, and provocative of waste. The employment of alcohol, tea, coffee, etc., would tend to correct this undesirable acceleration of the assimilative processes; for Sir W. Roberts has proved, by a series of carefully conducted experiments, that their effect is powerfully to retard the action of the various digestive ferments on the foods; and it may be that the instinctive sense of the benefit thereby derived lies at the root of the yearning of all civilised nations for such substances. Again, some condiment, such as common salt, is added, to restore sapidity to articles from which the salts have been removed in the process of cooking; and, taken in excess, it only throws extra work on the organs of excretion. (*British Medical Journal* 1886;i:126.)