Fig. 1. Southern blot analysis of cervical cancer biopsies. Cellular DNAs (10 \( \mu \)g each) were digested with Bam HI (for HPV-16) and EcoRI (for HPV-18) and electrophoresed in a 0.8% agarose gel and blotted onto hybond nylon membranes. Hybridization was done at Tm-20 °C as described in the text using vector free 32p—labelled HPV-16 (a) and HPV-18 (b) DNA probe respectively. Lane 7 in Fig. 1b is HPV-18 DNA (7.9 kb) (0.1 M NaOH and 1.5 NaCl) for 1 h at 42 °C followed by neutralization with 1.5 M NaCl, 1.0 M Tris Cl. (pH 8.0) of 4 h at 42 °C.

HPV-DNA sequences were detected in 33/40 (82.5%) biopsy specimens and HSV-2 DNA sequences were found in 4/40 (10%) biopsies (Table I, Fig. 1). HPV-16 DNA sequences alone were present in 30% biopsies and in another 20% biopsies along with HPV-18 sequences. Whereas, HPV-18 DNA sequences alone were detected in only 15% biopsies. All the four biopsies which had HSV-2 Bgl II N fragment DNA sequences were also positive for HPV-16 and/or HSV-18 DNA sequences. In 4/40 biopsies HPV-types other than HPV-16/18 were present.

A wide variation in the distribution of HPV-DNA sequences of different HPV-types in cervical carcinoma from various parts of the world has been observed [1]. Nevertheless, HPV-16/18 account for more than two thirds of HPV positive specimens. It would be of interest to see whether the HPVs other than HPV 16/18 found in the present study represent additional HPV types (i.e. HPV 6/11, 31, 33, and 35) less commonly seen in cervical cancers or whether novel types occur in Indian population.

Recently a few studies have examined the distribution of HPV DNA according to various histologic types of cervical cancer. Tase and his associ...
(personal communication) have detected only HPV-16 DNA in all HPV positive squamous cell carcinoma and only HPV-18 DNA in almost all HPV positive adenocarcinoma of cervix. However, in adenosquamous carcinoma, HPV-16 and 18 were equally distributed in HPV positive biopsies. Similarly, Ikenberg et al. [pers. comm.] found predominantly HPV-16 DNA in squamous cell carcinoma with very few biopsies positive for HPV-18. Whereas, in adenocarcinoma majority of biopsies contained HPV-DNA sequences other than HPV-16/18.

In the present study, analysis of data according to histologic type of cervical cancer shows that in 25% keratinizing squamous cell carcinoma HPV-16 alone was present and in 10% biopsies HPV-18 DNA alone was found, whereas HPV-16 as well as 18 DNA was detected in another 25% biopsies (Table 1). On the other hand, in biopsies showing nonkeratinizing undifferentiated squamous cell carcinoma, 38.9% had HPV-16 DNA only, 16.7% had HPV-18 only, 11.1% had HPV-16 and HPV-18 and 11.1% had HPVs other than HPV-16 and 18.
The number of biopsies from adenocarcinoma is too few to draw any conclusion. However, no significant correlation seems to exist between HPV-types and histologic types of grade of differentiation of tumor. Similarly, HSV-2-Bgl II-N fragment DNA sequences were present only in 4/40 (10%) biopsies: two were with keratinizing squamous cell carcinoma and the other two were with non keratinizing undifferentiated type. All the four biopsies had HPV-16 and/or HPV-18 DNA sequences as well. This presence of HSV-2 DNA in cervical cancer may be a coincidental finding.

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Detection of herpes simplex virus type 2 DNA in uterine cervix lesions using cloned Bgl II N fragment of HSV-2 DNA as a probe

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Bgl II N fragment (transforming fragment, mu 0.58—0.62) of the DNA from a local isolate of herpes simplex virus type 2 (HSV-2) (HV 219 strain) was cloned in pKC-7 plasmid vector. The Bgl II N fragment specificity of this clone was confirmed in a spot hybridization assay using subgenomic clones of another strain of HSV-2 (strain 333) in pHC-79 cosmid vector. The cloned HV 219 Bgl II N fragment was made vector free and nick translated using 32P dCTP. This probe was used to screen cervical biopsies from 23 patients with invasive cervical carcinoma and 11 patients with chronic cervicitis for the presence of HSV specific DNA sequences in a southern blot hybridization assay. Hybridization was seen in 1 of 11 (9.1%) chronic cervicitis patients and 2 of 23 (8.7%) patients with invasive cancer, thereby suggesting that the presence of HSV-2 DNA in cervical cancer may only be a coincidental finding.

Herpes simplex virus (HSV) is a large DNA virus containing a double stranded DNA of approximately 160 kilobase pairs (Kb) size. The viral DNA, including subgenomic fragments, can induce malignant transformation of mammalian cells in vitro. Two regions in the herpes simplex virus type 2 (HSV-2) DNA spanning between map units (mu) 0.58-0.62 and 0.43-0.58 (representing N and C fragments respectively of Bgl II digest of HSV-2 DNA) have been shown to transform cells independently in vitro.

Evidence associating HSV-2 with uterine cervical cancer came mainly from sero-epidemiological studies from different parts of the world which showed higher incidences as well as titres of HSV-2 antibodies in cervical cancer patients as compared to matched controls. At a molecular level this association has not been corroborated consistently. Though initially Frankel and co-workers reported the presence of HSV-2 DNA in the cervical cancer tissue, several workers failed to confirm these findings.
However, recently using cloned subgenomic DNA fragments as probes, HSV-2 specific sequences have been detected in about 20 per cent of cancer tissues\textsuperscript{9–11}. In the present study, Bgl II N fragment of DNA (mu 0.58–0.62) from a local strain of HSV-2 (HV-219)\textsuperscript{12} was cloned in pKC-7 plasmid vector and used as hybridization probe to investigate the presence of HSV-2 DNA in non-malignant and malignant lesions of uterine cervix.

**Material & Methods**

*Cell and virus*: African green monkey kidney cells (Vero) were grown in MEM containing 5 per cent calf serum, 1.76 \( \mu g/ml \) NaHCO\(_3\) and antibiotics (penicillin 100 units/ml, streptomycin 100 \( \mu g/ml \) and kanamycin 25 \( \mu g/ml \)). HV-219 strain of HSV-2\textsuperscript{12} was used for cloning the DNA.

*Extraction of viral DNA*: Viral DNA was extracted from HSV-2 infected Vero cells as described by Lonsdale et al\textsuperscript{13}. Briefly, Vero cells were infected with HSV-2 at a multiplicity of infection of 5 pfu/cell. Once a complete cytopathic effect appeared the cells were harvested, washed with phosphate buffered saline (PBS), pH 7.2, and suspended in lysis buffer containing 10 mM Tris HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl\(_2\) and 0.5 per cent NP-40 (20 ml for 1 \( \times 10^6 \) infected cells). The mixture was incubated on ice for 10 min and cellular nuclei were pelleted at 2000 \( g \) for 10 min at 4°C. The supernatant containing virus particles was ultracentrifuged at 80,000 \( g \) for 1 h at 4°C (Sorvall, DuPont, USA). The pellet was resuspended in 2.5 ml of a solution containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 2 per cent SDS. To this was added an equal volume of phenol (saturated with 75 mM NaCl and 50 mM EDTA) and the mixture was incubated on ice for 10 min. Following centrifugation at 2000 \( g \) for 10 min, the aqueous phase was removed and extracted with equal volume of chloroform (24 : 1, chloroform : amyl alcohol) and then treated with pancreatic RNase A to a final concentration of 20 \( \mu g/ml \). The mixture was dialysed with several changes of buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM NaCl and 1 mM EDTA. The concentration of HSV-2 DNA was determined by spectrophotometer (Shimadzu, Japan) at 260 nm.

*Restriction enzyme digestion of DNA and elution of N fragment*: 100 \( \mu g \) of HSV-2 DNA was digested with 100 units of Bgl II (Bethesda Research Laboratory, USA) as per manufacturer's instructions at 37°C for 1 h. The digested DNA was electrophoresed in a 0.8 per cent low melting agarose gel (Type VII, Sigma Chemical Co, USA) at a constant voltage of 2 v/cm for 15 h using appropriate molecular weight markers. The gel was stained with ethidium bromide (0.2 \( \mu g/ml \)) and the DNA fragments were visualised on a UV transilluminator (Fotodyne Inc., USA). The part of the gel containing a 7.6 Kb fragment (representing Bgl II N fragment) was cut out and the DNA was eluted from low melting agarose as described by Maniatis et al\textsuperscript{14}. The DNA fragment was melted at 65°C for 15 min and extracted with phenol/chloroform. The DNA was precipitated with ethanol and suspended in a small volume of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA.

*Cloning of N fragment*: Escherichia coli, HB 101 containing pKC-7 plasmid (a pBR derivative with a single Bgl II site)\textsuperscript{15} was grown in Luria broth (10% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl). The plasmid was extracted
sC alkaline lysis method and purified on byCl gradient\textsuperscript{14}. Twenty micrograms of pKC-7 plasmid was digested with Bgl II restriction endonuclease, purified by phenol/chloroform extraction and ethanol precipitation.

One microgram of Bgl II digested pKC-7 DNA and 1 \mu g of HSV-2 Bgl II N fragment DNA were ligated in a ligation mixture containing 0.5 M TrisHCl, pH 7.4, 0.1 M MgCl\textsubscript{2}, 0.1 mM dithiothreitol, 10 mM spermidine, 10 mM adenosine triphosphate, 1 mg/ml bovine serum albumin and 20 units of T\textsubscript{4} DNA ligase (CSIR Centre for Biochemicals, Delhi) at 12°C for 15 h. HB\textsubscript{101} strain of Esch. coli was transformed using the ligated DNA as described by Maniatis \textit{et al}\textsuperscript{14}. The transformants were screened for the presence of Bgl II N fragment of HSV-2 DNA by restriction analysis and spot hybridization using a reference subgenomic clones of another HSV-2 (strain 333)\textsuperscript{16} in pHC-79 cosmid vector (gifted by Dr Sylvia Bachetti, McMaster University, Canada).

\textit{Probe}: Recombinant pKC 7 plasmid containing HSV-2 Bgl II N fragment was digested with restriction enzyme Bgl II and the vector free Bgl II N fragment was eluted following electrophoresis using low melting agarose\textsuperscript{14}. This vector free N fragment was nick translated using nick translation kit (BRL, USA) and \textsuperscript{32}P dCTP (DuPont-NEN, USA) to a specific activity of 0.5 to 1 x 10\textsuperscript{8} cpm/\mu g DNA.

\textit{DNA from cervical biopsies}: Colposcopically directed cervical biopsies were taken from patients attending the cancer clinic of the Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi. Twenty three and 11 histopathologically confirmed patients of invasive cancer and chronic cervicitis respectively were studied. The biopsies were stored at -70°C before processing.

DNA was extracted from the biopsy samples essentially as described by Prakash \textit{et al}\textsuperscript{11}. Briefly, the biopsy tissues were washed with PBS, minced thoroughly with scissors and digested with pronase (500 \mu g/ml) for 24 h at 37°C in STE buffer (50 mM TrisHCl, pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% SDS). The digested samples were twice extracted with distilled phenol (saturated with 100 mM TrisHCl, pH 8.0 and 10 mM EDTA) and the aqueous phase was collected and extracted twice with anhydrous ether and the DNA was pelleted, dried under vacuum and dissolved in a TE buffer (10 mM TrisHCl, pH 8.0, 1 mM EDTA). The DNA from each biopsy sample was digested with EcoRI under reaction conditions recommended by the manufacturer (BRL, Maryland, USA). Ten micrograms of restricted DNAs were separated on 0.8 per cent agarose gels at a constant voltage of 2 v/cm for 15 h. The separated fragments were transferred\textsuperscript{14,16} onto Hybond nylon membranes (Amersham, UK). After completion of transfer the membranes were baked at 80°C for 2 h in a vacuum oven, individually sealed in plastic bags and stored at 4°C till processed.

\textit{Hybridization}: Hybridization reaction was carried out essentially as described by Maniatis \textit{et al}\textsuperscript{14}. Southern blots were prehybridized in a solution containing 50 per cent v/v deionized formamide, 5 x SSC, 5 x Denhardt's reagent (0.1% w/v Ficoll, 0.1 % w/v polyvinyl pyrrolidone, 0.1 % w/v bovine serum albumin), 200 \mu g/ml denatured salmon sperm
DNA, and 50 mM TrisHCl, pH 7.5 for 5 h at 42°C. Hybridization was carried out in the same solution in the presence of heat denatured probe (1 x 10^6 cpm/ml) for 24 h at 42°C. The blots were then washed twice for 5 min and once for 15 min at room temperature in 2 x SSC containing 0.1 per cent SDS. Another 2-3 washes were given in the same buffer at 65°C (1 h/wash), following which additional 2 washes were given with 0.1 x SSC and 0.1 per cent SDS at 65°C (1 h/each). The membranes were then dried at room temperature and exposed to superspeed polyester X-ray films (Indu, Hyderabad) with intensifying screens for 10 days at -70°C.

Results

Since the HSV-2 Bgl II N fragment was cloned in pKC-7 plasmid, the recombinant plasmid should release the insert DNA following digestion with Bgl II enzyme. The transformants were analysed by this method and a transformant containing a recombinant plasmid which released a 7.6 Kb fragment upon digestion with Bgl II (the size of HSV 2 Bgl II N fragment) was selected for further study. To verify the HSV-2 Bgl II N specificity of this 7.6 Kb insert, the vector free insert DNA was labelled with 32P and used as a probe to screen the reference HSV-2 (strain 333) recombinant clones in pHC 79.

Fig. 1 Spot blot hybridization assay showing Bgl II N specificity of the recombinant clone using the insert as a probe to hybridize a reference HSV genomic library of Canada (strain 333 of HSV-2 in pHC-79 vector). 10 ng DNA from each of the reference clones containing Bgl II fragments C + N + I + H + K (A), D + R + P + G + J (II), G + I + O + C (C), H + M + Q + L + K (D) and genomic HSV-2 DNA (E) were spotted on to nitrocellulose filter, denatured in situ and hybridized using 32P labelled vector free 7.6 Kb insert DNA.
cosmid vector each of which contained different Bgl II fragments of HSV-2 DNA. Hybridization was seen with whole HSV-2 genomic DNA and pHC 79 clone containing N fragment but not with pHC 79 clones lacking the N fragment (Fig. 1) confirming thereby that our recombinant clone in pKC 7 plasmid contains HSV 2 Bgl II N fragment.

Biopsies from 23 patients with invasive cervical carcinoma and 11 patients with chronic cervicitis were investigated. One of eleven (9.1 %) biopsies from chronic cervicitis patients and 2 of 23 (8.7 %) from invasive carcinoma contained HSV-2 Bgl II N fragment specific sequences (Fig. 2, lanes 2,3).

Discussion

Despite a serological association between HSV-2 and cervical cancer, attempts to detect viral DNA in cervical carcinoma tissues were generally unsuccessful. Failure to detect HSV-2 DNA might have been due to the low sensitivity of the probes used by these workers. More recently using cloned subgenomic fragments of HSV-2 DNA, several workers have independently reported the detection of viral DNA in 15-20 per cent of biopsies by Southern hybridization technique. Generally, the region of HSV-2 DNA hybridizing with cervical cancer biopsies has been located at 0.58-0.62 mu (Bgl II N fragment). Since this fragment readily transforms the cells in vitro and also produces tumors in nude mice as compared to Bgl II C fragment (0.43-0.58 mu) which only induces morphological transformation at a low efficiency in a continuous passage assay, we decided to clone and use N fragment of Bgl II digested HV-219 strain of

Fig. 2 Southern blot analysis of cervical cancer biopsies. Cellular DNAs (10 mg each) were digested with EcoRI, electrophoresed in 0.8 per cent agarose gel and blotted on to Hybond nylon membrane. Hybridization was done at Tm-20°C as described in the text using vector free 32P labelled HSV-2 Bgl II N DNA probe. Lane 1 : HSV positive control at one genome equivalent. Lanes 2 and 3 : cervical cancer biopsy samples.
HSV-2 (a local isolate from India) for hybridization. Using this cloned fragment, we observed that 1 of 11 (9.1%) biopsies from cervicitis patients and 2 of 23 (8.7%) from invasive cancer patients contained HSV-2 specific sequences. These findings suggest that the presence of HSV-2 DNA in cervical malignancies is perhaps a coincidental finding and the virus may not have a role in its causation. On the other hand, recently Galloway and colleagues observed that HSV-2 specific sequences as well as specific RNA transcripts were absent in the cells transformed in vitro by the viral DNA and suggested that HSV may be causing malignant transformation of the cells by a 'hit and run' mechanism. Further, deletion analysis of Bgl II N fragment has led to the identification of a 737 base pair fragment located in the N fragment which could transform NIH 3T3 cells in vitro. Interestingly, the sequence of this fragment is such that it could be drawn as a stem loop structure flanked by direct repeats rather resembling insertion sequence like elements. Activation of cellular oncogene expression by insertion sequence transposition has also been suggested as a possible alternative mechanism of HSV induced transformation. The presence of such small sequences might go undetected in routine hybridization assays.

On the other hand, zur Hausen has suggested that HSV might not be the sole etiological agent in cervical carcinogenesis. It could be playing a role of a co-factor in this process. Indeed a number of recent reports have incriminated human papilloma virus (HPV) in the etiology of cervical cancer and there has been a suggestion that HSV might synergistically interact with HPV in the induction of carcinogenesis.

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