DISCUSSION
Epidemiological study

In accordance with most of the epidemiological studies on cervical neoplasia, the present study reveals the occurrence of this disease in sexually active women. Although women with multiple sexual partners are considered to be at higher risk of developing cervical cancer (Zunzunegui et al., 1986; Brinton et al., 1987) this information in the present study could not be obtained due to social constraints.

Majority of the women in the present study had multiple pregnancies (4+), a factor considered to be important regarding the etiology of cervical cancer. In an epidemiological study on patients with cervical cancer, Brinton et al. (1987) have also placed such women at high risk of developing cancer along with those exposed to sexual activities below the age of 15 years (2 to 4 folds elevated risk) than those at the age of \( \geq 19 \).

The age of the patients in this study ranged from 21-80 years with peak incidence between 40-60 years. It, thus, appears that cervical carcinoma is not essentially a post-menopausal disease. In a study on squamous cell carcinoma, adeno-squamous carcinoma and endometrial adeno-carcinoma, only the last one has been identified as a post-menopausal disease. The mean age of patients with squamous cell carcinoma was comparatively less and no relationship with menstrual factors like age at menarche, regularity of menstrual cycle and age at menopause seemed to exist.
The role of oral contraceptive could not be ascertained in this study as none of the respondents was user of pills for birth control. There is, however, lack of consistency regarding the role of oral contraceptive in the etiology of cervical carcinoma. It has been considered as a high risk factor by Brinton et al. (1986) and Ebeling et al. (1987) while others could not find the use of oral contraceptive significant in this regard (Celentano et al., 1987; Molina et al., 1988; Acs et al., 1989). The use of oral contraceptive has been found unrelated to the risk of atypia or low grade squamous intra-epithelial lesions (SILs) but has association with high grade SIL which increased with the longer duration of use ( > 5 years). However, no synergistic action of oral contraceptive and Human papilloma virus infection could be seen by Negrini et al. (1990). Marginally elevated risk of invasive carcinoma among women with long term oral contraceptive use has been reported by Brinton et al. (1990). In the subjects of present study the basic concept of contraception or family planning was lacking. Majority of them did not use any contraceptive method, a fact also evident from the number of children borne to them.

The lack of early screening was evident from this study as maximum number of cases were at stage III of cervical neoplasia at the time of diagnosis. The lack of Pap smear screening is a major risk factor (Brinton et al.,
The women with carcinoma in situ of cervix have less than 1% chance of death from cancer in five years following diagnosis in contrast to almost 97% risk of death in cases with metastatic cervical cancer. The possible decline in cervical cancer mortality in the developed countries is mainly due to the widespread use of cytological screening for early diagnosis and treatment (Miller, 1985). It is thus suggested that regular screening of not only the post-menopausal but also the sexually active women in any age group is important for the management of cervical cancer.

Majority of the respondents in this study belonged to low income group coming from the rural areas. Although the women with low socio-economic background are at increased risk of developing cervical cancer as compared to middle and upper classes (Brinton et al., 1987; Marcus et al., 1990), the specific risk factors are not well marked out. Nutritional deficiencies, unhygienic conditions, non-availability of primary screening facilities, different health care practices and low educational level may be responsible for the high risk. In this study maximum number of patients were from the state of Punjab because PGIMER, Chandigarh from where the samples were taken is a referral centre for the state.

Regarding the medical history of patients, only a few cases in the present group had diabetes and no significant association was seen with any kind of pre-existing disease in them. A similar observation has been made by Brinton et
al. (1987) with regard to association of pre-existing diseases like hypertension, diabetes, gall bladder or thyroid diseases with the risk of cervical tumour.

These observations presented with a small sample provide a nucleus for the further detailed etiological evaluation of risk factors for cervical cancer by considering multiple parameters based on circumstantial evidence and experimental studies. The latter require the molecular analysis to screen for the possible association of Human papilloma virus with such cases and its synergistic association with any of the risk factors. However, conclusions can only be drawn from case control studies with large sample size.

Cytogenetic analysis

The current study on the patients with cervical carcinoma showed significant elevation in the frequency of SCEs (P<0.01) and chromosomal aberrations (P<0.05) in the lymphocyte culture over those of controls. These observations are in coherence with those of Mitra et al. (1982) and Murty et al. (1986) with regard to SCEs and Sobti et al. (1991) on chromosomal aberrations in the patients with cervical tumours but are in contrast with the observations made by Adhvaryu et al. (1985). There are reports that the cancer affected beings, including man, reveal an elevated frequency of SCEs and CAs and notable amongst these are reports on cancer bearing mice (Banerjee et al., 1982) and fish (Park and Grimm, 1982) which show an increased
frequency of SCEs in bone marrow cells or peripheral blood lymphocytes. A similar picture has been observed in patients with malignant lymphoma (Kurvinik et al., 1978), acute lymphoblastic leukemia (Otter et al., 1979) and carcinoma of lung (Hopkin and Evans, 1980). However, certain contrary reports such as patients with malignant lymphoma (Crossen et al., 1981), chronic myelogenous leukemia (Cheng et al., 1979) and chronic lymphocytic leukemia (McDonald and Fitzgerald, 1979) in which no change in the SCE levels has been observed are available. Thus there is a lack of consistency in these findings.

The critical analysis of the data given in the paper of Adhvaryu et al. (1985) makes it apparent that though SCEs have not proven to be statistically significant, there is an upward trend in most of the cases. Also the number of cases taken (n=13) was quite small to support the generalized conclusion that the presence of carcinoma has no bearing on the frequency of SCEs in peripheral blood lymphocytes.

The current study witnessed an upward trend in the frequency of SCEs with regard to the stage of cancer. However, the number of patients analyzed at stages I and II were low as compared to those at stage III. A similar report has been published by Yokota et al. (1989) where it has been concluded that the frequency of SCEs in patients with cervical cancer is related to the presence of cancer but is not a predisposing factor.

The present data on the TCAs in these patients
revealed decrease in the frequency of aberrations with the advancement in the stage of cancer and the values were lowest in patients at stage III. The fall in the values of CAs was due to the decrease in the value of chromatid type aberrations (Table-2.7) that might have got repaired by the cells while they pass through the 'S' phase of cell cycle which may also be favoured by the prolonged cell cycle in these patients (Table-2.13). In addition, the cells with drastic aberrations such as dicentrics or a lethal break or exchange might have been knocked out of the population leading to the formation of cell population that was stabilized in the new environment i.e. the presence of carcinoma. This phenomenon has also been supported by the cytogenetic analysis of the epithelial cells immortalized by HPV-16. The high degree of chromosomal alterations, reflecting genomic instability, diminished with several passages as a population with stable structural alterations emerged (Popescu et al., 1989).

It is suspected that there is some correlation between the elevated levels of chromosomal aberrations and of SCEs in the patients with carcinoma of cervix and some other cancers but the exact significance with regard to the degree of damage and type of cancer is still to be worked out. Sobti et al. (1991) have indicated that the genome of patients with cervical tumour is fragile and this is also evident from the observations of Adhvaryu et al. (1985) and Yokota et al. (1989) who have seen a significant increase in
SCE levels in lymphocytes of cancer patients treated with mutagen, mitomycin-C, and increased chromosomal fragility with clastogen, aphidicolin (Paz-y-Mino et al., 1992).

The frequencies of SCEs and TCAs may vary in relation to a large number of factors such as age, sex, genetic make up of the individual or environmental factors like smoking, alcohol intake, radiation or chemical exposure. The patients included in the current study were not exposed to radiotherapy or chemotherapy and none of them were smokers or alcoholics or users of oral contraceptive. The evaluation of relationship between age and the SCEs revealed that the frequency of SCEs was independent of the aging factor while TCAs showed a positive correlation with age but the value of correlation was insignificant. Similar correlation has also been reported by Yokota et al. (1989) and Livingston et al. (1983).

No correlation was found between the frequency of SCEs and the TCAs in the patients as well as in the controls. Our observation is in accordance with the findings of Hedner et al. (1982) according to whom SCEs and chromosomal aberrations probably arise by partly different mechanisms and reflect different kinds of primary DNA damage. Yokota et al. (1989) have also proposed that an increase in frequency of SCEs in patients with cervical cancer is attributable to a mechanism essentially different from that of specific oncogene or chromosomal aberration.

The elevation of the frequencies of SCEs and CAs in
the cancer patients with the increase in the number of pregnancies could be due to the immunosuppressive state (albeit temporary) or due to the hormonal changes during pregnancy that may favour the growth of tumour which may be reflected in the form of increased levels of SCEs and CAAs.

Various possible mechanisms have been proposed to explain the increase in the level of SCEs and the chromosomal aberrations in cancer patients. The metabolic stress on the system due to the tumour growth (Banerjee et al., 1982) or the clastogenic products released by the tumour cells (Werkmesiter et al., 1980; Park and Grimm, 1982) may be responsible for the elevated level of SCEs. Another factor which can be of significance is the presence of virus. Higher SCE frequencies in the peripheral blood lymphocytes have been reported in European eels with cauliflower tumour which could be due to a virus (Park and Grimm, 1982). Kurvinik et al. (1978) have also highlighted the presence of viral infection in cases with increased levels of SCEs. The viral integration into the cell genome produces no specific cytogenetic manifestations. The general changes are altered mitotic indices, polyplody, gaps, breaks and abnormal chromosomal figures (AbuBaker et al., 1988; Chieco-Bianchi et al., 1988; Popescu et al., 1990).

Herpes simplex virus-2 (HSV-2) and Human papilloma virus (HPV) have been extensively studied with regard to their roles in the genesis of cervical cancer. In a study on such patients, Ghosh and Ghosh (1983) have shown a
positive correlation of HSV antibody titre with an increased frequency of SCEs. However, Murty et al. (1986) made contrary observations in relation to the HSV antibody titre in serum. In a recent study on women with cervical carcinoma, Paz-y-Mino (1992) have indicated the relationship of the level of chromosomal fragility vis-a-vis the presence of Human papilloma virus that has been implicated with 98% of the preneoplastic and neoplastic lesions of cervix (Das et al., 1992).

The current study on the incidence of HPV in cervical cancer patients (as revealed by DNA hybridization analysis) also projects the presence of HPV in 88% of the cases (under relaxed conditions of hybridization with HPV-16 DNA probe) which may be responsible for the elevated levels of SCEs and TCAs in patients with cervical carcinoma. Since the level of chromosomal fragility also varies with the type of HPV involved (Paz-y-Mino et al., 1992), it has thus been proposed that it may be used as a cytogenetic marker reflecting the evolution, prognosis and treatment of women infected with HPV. The relationship between the genomic fragility and HPV infection is also evident from the studies of Popescu et al. (1990) and Haas and DeBraekeleer (1989) where HPV integration sites were found to lie near or coincided with chromosomal fragile sites, cancer chromosome break points and location of oncogenes.

The analysis of cell cycle kinetics revealed the accumulation of cells at M1 phase. The accumulation was
higher in the patients and likewise there was a lower number of cells going through M3 phase. This indicates a longer cell cycle of the lymphocytes in patients as compared to the controls. Longer cell cycle duration has also been found in leukemic cells (Abe and Sandberg, 1980). A similar trend has been observed in lymphocytes of patients with acute lymphoblastic leukemia (Heerema et al., 1982). Our observations are, however, in contrast to those of Adhvaryu et al. (1985) according to whom there is no effect on the cell cycle in response to the cervical cancer. However, they have referred to the possibility of obtaining some useful information by employing more than one inducing agent (in addition to mitomycin-C) at different concentrations on a larger population.

The prolongation of cell cycle seems to be related to the severity of the disease, as is evident from the increase in the number of cells at M1 in patients at stage I and II as compared to the controls. The fall in the M1 cells in patients at stage III could be due to the stabilization of the lymphocytes to the presence of carcinoma.

Immunohistochemical analysis

Papilloma viruses can infect avian, reptile and mammalian hosts and humans are susceptible to more than 60 types. The major capsid protein (L1) is the most abundant viral protein which is highly conserved among different papilloma virus types. Immunological relatedness of papilloma
viruses from different species as demonstrated by Jenson et al. (1980) has thus made it possible to detect genus specific antigens immunologically. Genus specific antigens of papilloma virus are generally found in koilocytic and flattened superficial squamous cells in a well differentiated tissue (Ferenczy et al., 1981; Fu et al., 1981; Alessandri et al., 1986). The expression of papilloma virus genes is linked to the state of keratinocyte differentiation (Reid, 1984). In invasive carcinoma the loss of differentiation may be unfavourable for the maturation of virus resulting in non-detection of viral antigens despite the presence of virus.

Kurman et al. (1981) showed the presence of viral antigen in 50% of the biopsies of cervical dysplasia and vulvar condyloma. The number of HPV antigen positive cases has been shown to increase in the cases of laryngeal papilloma from 50 to 90% and then to 100% by analyzing sequential biopsies over several months (Lack et al., 1980). This suggested that sampling error or periodic expression of the mature virus may be responsible for low detection rates. It has also been suggested that HPV-16 may produce little capsid antigen which escapes immunohistochemical detection (Beckmann et al., 1988).

The negative results of the immunohistochemical analysis of patients in this study can be explained on the basis of the fact that all the biopsy samples were from patients with invasive carcinoma of cervix (Stage I-IIIb).
The decrease in the number of antigen positive cells with the increase in the severity of lesion is well documented by studies of the cases of mild dysplasia, carcinoma in situ and invasive carcinoma of cervix (Syrjanen and Pyrhonen, 1982; Sato et al., 1987). This has also been supported by Hara et al. (1990) where the detection rate of HPV-16 was highest in differentiated keratinizing type of cervical carcinoma as compared to small cell non-keratinizing type.

It may thus be hypothesized that it is the persistence of viral genome rather than the productive viral infection that is associated with the carcinogenic phenotype. It is also evident from the observation that 3/4 vulvar warts, though negative for HPV capsid antigen, were positive for HPV-DNA (Henderson et al., 1987). There are reports that lesions that were negative for HPV infection when examined by electron microscopy or immunohistochemistry were demonstrated positive with nucleic acid probes (Pfister, 1984; Syrjanen, 1986). This has been further supported by the observations of Gupta et al. (1987) where cases positive for HPV-antigen belonged to no-CIN and CIN-I group while HPV infection was best documented by HPV-DNA probes in CIN-II and CIN-III groups. Thus an attempt was made, in this work, to study the occurrence and incidence of HPV by molecular DNA hybridizations based on the presence of HPV-DNA in the samples.
DNA hybridization analysis

*E. coli* HB101 was transformed with HPV-16 DNA cloned in *Bam HI* site of pBR322 (HPV-16 : pBR322) resulting in the insertional inactivation of tetracycline resistance (*tet*), but intact ampicillin resistance (*bla*) gene. Thus *E. coli* cells transformed with HPV-16 : pBR322 were tetracycline sensitive (*Tc^s*) but were able to express ampicillin resistance (*Ap^r*) and these characters were used as markers for the selection. The low efficiency of transformation was probably due to the large size of plasmid (~12.3 kb) as the efficiency of transformation is inversely related to the size of the plasmid and becomes a limiting factor when plasmid size exceeds 15 kb (Sambrook et al., 1989).

The digestion of plasmid DNA, obtained from the transformant, with *Bam HI* and *Eco RI* confirmed the presence of HPV-16 : pBR322. The interpretation was based on the fact that pBR322 has a single site both for *Bam HI* and *Eco RI* (Sambrook et al., 1989) and HPV-16 has a single site for *Bam HI* but two for *Eco RI* (Choo et al., 1989).

For the molecular hybridization studies, vector-free HPV-16 DNA was used as a probe so as to eliminate the chances of false positive results which may crop up due to any bacterial contamination of the biopsy samples. The contaminating bacteria may contain pBR322 or its related sequences that may hybridize with the vector pBR322.

The incidence of HPV in most studies has been worked out with molecular hybridization techniques using radio-
labelled probes. In the present study the dot blot and Southern blot hybridization analyses were done with biotin-labelled probe which is safer to use, less expensive, gives quick results, has a long shelf life and has been reported to be comparable to radiolabelled probes regarding sensitivity. With biotinylated probe the use of nitrocellulose paper was preferred over the nylon membrane since the latter showed lot of noise (background signal) which interfered with the ease and sensitivity of detection. In general also, nylon membranes are known to give much more noise than the nitrocellulose paper (Sambrook et al., 1989).

The results of dot blot hybridization under relaxed conditions revealed the association of HPV-DNA in 88% of the invasive carcinoma cases and 80% of those showing abnormal cervical cytology while only 40 and 20% of the cases were specifically positive for HPV-16 DNA in both the groups, respectively, under high stringency conditions of hybridization. The additional positive signals (48 and 60% respectively) under low stringency conditions may be due to the presence of HPVs other than type-16 but having a homology (consequently cross-hybridizing) with the latter.

Additional positive signals under low stringency conditions of hybridization have also been reported by Durst et al. (1983). The cross-reactivity of HPV-16 DNA to HPV-31 and 33 has been observed (Stoler and Broker, 1986) which is also supported by the 70% homology between the
nucleotide sequences of HPV-16 and 31 genomes (Goldsborough et al., 1989). In addition, it has been pointed out by Sutton et al. (1987) that a positive signal in a dot blot does not mean that HPV-DNA in the biopsy specimen should be identical along its entire length to the probe DNA. However, the dot blot analysis using probes specific for other HPV types (HPV-18, 31, and 35 that are commonly associated with invasive carcinoma) will provide better information regarding the prevalence of various HPV types in this region.

It is evident from the literature that there is a lot of variation in the incidence of HPV. The detection of HPV varies with the geographical difference, focal heterogeneity of viral DNA replication within the lesion or more likely because of the difference in the sensitivity of the method of detection (Arends et al., 1990). The incidence of HPV-16 has been reported to be 34.8% in cervical cancer biopsies in Kenya and Brazil and 61% in Germany (Durst et al., 1983) while it has been 45% in England (Scholl et al., 1985). Dot blot analysis of the cases of precancerous dysplasia and invasive carcinoma has revealed incidences of 38 and 58% respectively (Shirasawa et al., 1986). Multiple probe analysis using HPV 6/11, 16, 18 and 31 has shown HPV-16 to be the most prevalent type in women with abnormal cervical cytology (Hallam et al., 1989). Filter in situ hybridization studies, supplemented with in situ hybridization results, have shown the incidence to be 33% (Ostrow et al., 1987)
while it has been 66% by Southern blot hybridization in another study on invasive carcinoma cases of cervix (Meanwell et al., 1987). In India, an incidence as high as 98% has been reported in cervical carcinoma cases by Das et al. (1992) using a highly sensitive technique like PCR and HPV-16 as the most prevalent type followed by HPV-18.

A study, conducted previously in this region, has shown the incidence of HPV-16 to be 40.4% and that of HPV-18 as 34.04% by dot blot analysis using $^{32}$P-labelled probes (Arora et al., 1991). Thus the incidence of HPV-16 obtained in our study, using biotin-labelled probe, matches with the previously reported one which also suggests that biotin-labelled probe is as efficient as the $^{32}$P-labelled probe with respect to the sensitivity.

Four of the specimens, positive for HPV-16 DNA by dot blot hybridization under high stringency conditions, were further subjected to Southern blot analysis. Due to the limited amount of DNA samples available from rest of the HPV-16 positive cases, the Southern blot analysis could not be performed on all the specimens which could have given an insight of the physical status of HPV-16 and its relation to the carcinogenic phenotype.

The Southern analysis for the physical status of HPV-16 suggested the presence of episomal form in two of the samples and integrated form in the other two. The DNA samples which on complete digestion with Bam HI resolved into a single band of ~ 8.0kb suggested the presence of
HPV-DNA in the episomal form since Bam HI, a single cut enzyme for HPV-16, linearizes the HPV genome to its unit length. The sizes of the bands in undigested and HindIII (a no cut enzyme for HPV-16) digested counterparts of these samples indicated that the episome is present in oligomeric forms although the degree of oligomerization was different in both the samples.

Similarly the DNA samples which did not yield the 8.0kb band on Bam HI digestion indicated the integration of HPV-16 into the cellular DNA. The off-sized fragments could be the viral-cellular DNA junctions. Since the bands were positioned at different places in these samples it was suggestive of random integration of HPV-16 into the cellular DNA. Independent and random integration pattern of HPV-16 has also been observed in a study on four human cervical carcinoma cases (Lehn et al., 1985). The inference on the physical status of HPV made in this study is mainly based on the guidelines outlined by Matsukura et al. (1989) and Das et al. (1992). Identification of integrated or episomal form of HPV can also be made by 2D gel electrophoresis particularly when both episomal and integrated forms are present in the same sample or when only a few sequences of HPV integrate into host DNA (Cullen et al., 1991).

In addition to 2D gel electrophoresis, PCR can also be employed to investigate the physical status of HPV. Using sequences specific for E2 open reading frame of HPV-16
as the primers, Das et al. (1992) have obtained results comparable to Southern blotting, 2D gel electrophoresis and chromosomal in situ hybridization. HPV-16 DNA was found in an integrated form in more than 70% of the cervical cancer tissues, 23% in severe dysplasia and carcinoma in situ while 2.5% cases showed both episomal and integrated forms. Fuchs et al. (1989) have detected episomal form of HPV-16 in 36% of the cervical cancer patients. On the other hand HPV-18 was found exclusively in integrated form. In another study on invasive carcinoma of cervix, 52% of the cases infected with HPV-16 DNA have been shown to contain integrated form, 20% had both integrated and episomal forms while 27% showed only the episomal form (Cullen et al., 1991).

It is, thus, apparent that both the episomal and integrated forms of HPV are associated with invasive carcinoma of cervix. Integration of HPV-DNA into the cellular DNA is generally taken as a prerequisite for malignant transformation (Durst et al., 1985; Shirasawa et al., 1986) which is further supported by the presence of integrated form of HPV in cell lines derived from cervical carcinoma (Tsunokawa et al., 1986; Shirasawa et al., 1987). The presence of episomal form alone in invasive carcinoma suggests that integration may be important but is not mandatory. In case of the episomal existence of HPV it is possible that rearrangements or mutations may result in functional alterations equivalent to the integrated state (Fuchs et al., 1989). It has also been suggested by Orth...
(1986) that episomal form of HPV-16 DNA in a cervical cancer may be sufficient by itself to introduce malignant phenotype as HPV-5 does in epidermodysplasia verruciformis.

For anogenital carcinogenesis by HPV, Zur Hausen (1991) has suggested that viral integration is essential. It mediates deregulation of expression of E6/E7 viral oncogenes due to the disruption of E2 open reading frame (in wake of integration) which encodes the transcriptional modulator proteins. In contrast, Nasseri et al. (1991) have observed that E2 gene product does not have significant effect on the expression of E7 protein in immortalized cervical keratinocytes. This has been further supported by the presence of E6/E7 transcripts of HPV-16 and 33 in tonsillar carcinoma independent of the physical status of the virus. It is thus evident that the maintenance of malignant phenotype is not dependent solely on the integration of HPV-DNA.