Interleukin 2 Production in Vitro by Peripheral Lymphocytes in Response to Human Papillomavirus-derived Peptides: Correlation with Cervical Pathology

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ABSTRACT

Human papillomavirus (HPV) is believed to be the major cause of cervical cancer. To investigate whether a cellular immune response, especially a T helper type 1 response, is related to the natural defense against HPV-related cervical lesions, the interleukin 2 response of peripheral blood lymphocytes in vitro to overlapping peptides from HPV-16 E6 and E7 oncoproteins was compared with the degree of cervical cytological abnormality among 140 women in a cross-sectional study. We compared 66 women diagnosed with low-grade squamous intraepithelial lesions (LSIL), 21 with high-grade squamous intraepithelial lesions (HSIL), and 28 with invasive cervical cancer with 25 women who were cytologically normal but previously HPV-16 DNA positive. The fraction showing strong interleukin 2 production against HPV-16 peptides was greatest among cytologically normal women (35%) and declined with increasing disease severity [LSIL] (20%), HSIL (17%), and cancer patients (7%); χ² test P for the trend = 0.02, whereas the responses against a recall influenza antigen were not significantly different among groups. Our finding suggests that a T helper lymphocyte type 1 response to HPV antigens is associated with disease status. This result may reflect a targeted effect of the disease on immune function or a protective effect of the immune response against disease progression.

INTRODUCTION

Infection with genital HPVs is common among sexually active individuals. There are over 30 HPV types known to infect the genital area and cause minor cytological abnormalities, including LSIL. A subset of these HPV types is known to be strongly associated with the development of cervical cancer, most notably HPV types 16, 18, and 31 (1-3). Although many women are exposed to these cancer-associated HPV types during their lifetime, most infections resolve spontaneously, and only a small fraction progress to HSIL and fewer yet to invasive cancer. Thus, other factors are likely to play a role in determining progression of HPV infection and low-grade lesions to high-grade lesions and cancer. These factors might be categorized into viral factors such as genotype variants, exogenous factors such as oral contraceptive use, and host factors such as the immune response.

Evidence suggests that the host immune response to viral infection is an important factor linked to the development of cervical neoplasia and that regression of low-grade HPV-related lesions may be mediated by a cellular immune response (4-11). Animal studies have demonstrated protection from infection and the development of neoplasia, as well as regression of existing lesions in immunized animals (5, 6, 10). Observational studies have documented an increased incidence of HPV-related diseases among transplant recipients and HIV patients, who are both known to have immunosuppressed cell-mediated immunity but normal humoral immune function (4, 7). Furthermore, studies have observed the infiltration of CD4+ (Th helper cells) and CD8+ (cytotoxic/suppressor T cells) T cells in spontaneously regressing warts, the depletion of antigen-presenting cells in the cervix of women with cervical cancer, and an association between specific HLA haplotypes and cervical disease risk, all of which suggest an important role for the cellular immune system in the host response to HPV infection and its low-grade lesions (9, 11-13).

If a cell-mediated immune response is important in the rejection of HPV infection and HPV-related cervical neoplasia, we hypothesized that a Th1 response (IL-2- and IFN-γ-producing) is likely to contribute to rejection. This hypothesis is based on work in a number of related fields, in which protection from chronic parasitic, bacterial, and viral diseases has been shown to be mediated by Th1 cells or cytokine responses and impaired by Th2 cytokine responses (14-25).

In the present study, we examined a Th1-type response among women with varying levels of cervical neoplasia and compared them to women who were known to have been infected with HPV but not to have developed evidence of low-grade cervical disease. This enabled us to specifically test out a priori hypothesis that successful immune responses would be those exhibited most frequently among infected women who remained cytologically normal and least frequently among women with high-grade cervical neoplasia and cancer. Th1-type responses were assessed by measuring IL-2 production in vitro in response to stimulation of donor PBMCs with HPV-specific antigens. As target viral antigens to evaluate T-cell responses, we chose the E6 and E7 oncoproteins of HPV-16 because HPV-16 is the most common viral type associated with cervical cancer (approximately 50% of tumors are HPV-16 positive; Ref. 2) and the E6 and E7 oncoproteins are retained at every stage of disease from the acute viral infection through squamous intraepithelial lesions to frank carcinoma (3, 26-28).
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PATIENTS AND METHODS

Patients. Subjects recruited into the present study were 140 women being seen at either one of the Kaiser Permanente clinics in Portland, Oregon, or the Simmons Cancer Center of the University of Texas Southwestern Medical Center in Dallas, Texas (Table 1). These 140 women correspond to 46.4% of a total of 302 women initially determined to be eligible for the study and represent a convenience sample of four different groups, including cancer, HSIL, LSIL, and women who remained normal despite HPV 16 infection. Eligible subjects recruited in Texas were 38 patients diagnosed with histologically confirmed invasive cervical cancer during the study period. In Oregon, eligible subjects represented 229 individuals referred to colposcopy due to an additional group of 35 cytologically normal women who participated in a large cohort study initiated in Oregon in 1989 (29). These latter subjects were all cytologically normal with no history of squamous intraepithelial lesions at the time of enrollment into the cohort (1989-1990) and were known to have been developing evidence of any dysplastic lesions. This control group was specifically selected to test the hypothesis that women who are infected with HPV-16 but do not develop cytological abnormalities are likely to mount a host immune response capable of adequately controlling HPV-16 infection, whereas women with advanced cervical neoplasia are likely not to mount such a response. Thus, by design, all study subjects were presumed to have been exposed to HPV infection. A control group comprised of women never exposed to HPV was not examined as part of this study for two reasons. First, our goal was not to compare immune responses to HPV antigens among exposed and unexposed women but rather to determine response among exposed women who developed disease versus those who did not. Second, it is extremely difficult to assemble a group of women known to never have been exposed to HPV, given the relative ubiquity of the virus among sexually active women (30). Even among sexually inexperienced women, vertical transmission (perinatal transmission) and nonsexual horizontal transmission is possible, and exposure to HPV can therefore not be ruled out (31).

Only 140 of the original 302 women determined to be eligible for study were included in the present analysis for the following reasons: (a) 44 women refused to participate; and (b) 118 subjects agreed to participate but assay results are unavailable because of delayed or unexpected receipt of the blood samples more than 2 days after shipment (n = 14), inadequate amount of viable lymphocytes recovered (n = 66), or technical problems encountered in the laboratory resulting from a faulty batch of human serum (n = 38).

A total of 30-70 ml of peripheral blood were collected from each study participant, kept at room temperature, and sent to the National Cancer Institute laboratory in Bethesda, Maryland via overnight air carrier. A cervicovaginal lavage sample (10 ml) was collected from all Portland subjects, with the sample more than 2 days after shipment (n = 14), inadequate amount of viable lymphocytes recovered (n = 66), or technical problems encountered in the laboratory resulting from a faulty batch of human serum (n = 38).

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Table 1 Characteristics of the study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>Normal*</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
</tr>
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<tr>
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<td>140</td>
<td>25</td>
<td>66</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Age (yr)</td>
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<td></td>
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<tr>
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<td>≥45</td>
<td>19%</td>
<td>8%</td>
<td>17%</td>
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<tr>
<td>Current HPV type%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HPV-16</td>
<td>44 (34%)</td>
<td>5 (20%)</td>
<td>17 (26%)</td>
<td>9 (43%)</td>
<td>13 (81%)</td>
</tr>
<tr>
<td>Other HPVs</td>
<td>25 (20%)</td>
<td>1 (4%)</td>
<td>15 (23%)</td>
<td>7 (33%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Negative</td>
<td>59 (46%)</td>
<td>19 (76%)</td>
<td>34 (51%)</td>
<td>5 (24%)</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>

* All normal women are known to have been HPV-16 DNA positive in the past 5 years, regardless of current HPV type.
% P for Mantel-Haenzel χ² for age by disease status = 0.001.
% HPV DNA data were available on tumor specimens from only 16 of the cancer patients. P for Pearson χ² for HPV by disease status = 0.001.

Peptides. Overlapping sets of mostly 15-mer (range: 10-16-mers) HPV-16 E6 and E7 peptides were prepared by the simultaneous multiple-antipeptide method of solid-phase peptide synthesis in polypropylene mesh "teabags" as described previously (32), based on the published sequences of HPV-16 E6 and E7 oncoproteins (33). Peptides were desalted by reverse-phase high-performance liquid chromatography on C18 Sep-Pak columns (Waters Associates, Milford, MA) and analyzed by high-performance liquid chromatography. Acetyl peptides were made for the HPV E6 oncoprotein. The positions of the peptides in the E6 and E7 sequences are shown in Fig. 1. Several peptides were insoluble and could not be studied (in E6, number 5, 8-11, 15-17, and 21; in E7, number 12 and 17). The soluble peptides used in the present study covered 88% of the total sequence of the E6 protein and the entire E7 sequence.

Stimulation of Human Peripheral Lymphocytes. PBMCs were separated from whole blood on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) and washed twice in PBS (Life Technologies, Inc., Grand Island, NY), then cultured by the method described (34), with modifications. Briefly, PBMCs (3 × 10⁵) were either unstimulated, stimulated with HPV peptides, or stimulated with FLU (FLU A/Bangkok RTX3 H3N2; 1:500 dilution) as a specificity control or PHA (PHA-M; Life Technologies, Inc.; 1:30 dilution) as a positive control in RPMI 1640 supplemented with 1% L-glutamine, 0.5% streptomycin-penicillin, and 10 mM HEPES at 37°C in a near 100% humidity 6% CO₂ incubator in 96-well plates in triplicate. The FLU antigen, as a single batch grown for this purpose, was stored at −70°C, and the optimum concentration was determined by preliminary experiments on PBMCs from healthy donors; the same lot was used throughout. The PHA was dissolved in sterile deionized water according to the manufacturer’s instructions and stored at −20°C. Human-type AB serum (at a final concentration of 5%; Sigma Chemical Co., St. Louis, MO) and anti-human IL-2 receptor antibody (anti-Tac; a kind gift from Dr. Thomas Waldmann, National Cancer Institute, Bethesda, MD) were added 1 h after the stimulation to prevent IL-2 consumption. On day 6, supernatants were harvested from each well, transferred into another 96-well plate individually, and stored at −20°C until assayed for IL-2.

Because of the limited number of PBMCs available for study, it was not possible to test each patient against the more than 40 peptides from E6 and E7 individually. Therefore, the peptides from each oncoprotein were divided into three groups of six to eight peptides each (Fig. 1), and each group was used for stimulation as an equimolar mixture. E6 group 1 (E6-G1) comprised the NH2-terminal third of the E6 protein, E6 group 2 (E6-G2) comprised the middle portion of the protein, and E6 group 3 (E6-G3) comprised the COOH-terminal third of the E6 protein. Parallel nomenclature was used for the E7 protein: E7 group 1 (E7-G1) represented the NH2-terminal side of the protein, and E7 group 3 (E7-G3) comprised the COOH-terminal side. At the concentrations of peptides used (5 μM), no substantial competition between peptides for binding to MHC is likely, and, in any case, should not affect the results because if any single peptide was positive within the mixture, the mixture was scored as positive, even if the positive peptide was interfering with the response to another one in the mixture.

IL-2 Assay. IL-2 in the culture supernatant was measured by the method described previously (34, 35). In brief, CTL-L2 cells (8 × 10⁵) were cultured in RPMI 1640 supplemented with L-glutamine, streptomycin-penicillin, 2-mercaptoethanol, HEPES, and 10% FCS (The Salzman Corp., Davenport, IA) with the supernatant described above at final concentrations of 1:2, 1:4, and 1:8.
each in triplicate. Twenty-four h later, 1 μCi of [3H]thymidine was added for the final 18 h. The cells were harvested with a 96-well cell harvester (Tomtec, Inc., Orange, CT), and the uptake of tritium into DNA was determined using a β-plate liquid scintillation counter (LKB, Uppsala, Sweden) as a measure of cell proliferation. This assay may potentially detect IL-15 as well, but because this does not affect the interpretation of our results, we refer to the activity as IL-2 for simplicity.

A sample was considered positive only if it met three separate criteria, each for at least two of the three dilutions (1:2, 1:4, and 1:8) of culture supernatant: (a) the mean [3H]thymidine incorporation (cpm) of each triplicate set had to be at least twice that of the medium control background; (b) the difference between the mean cpm of each triplicate set and the mean cpm of the medium control background had to be greater than 500; and (c) the experimental triplicate sets had to be statistically significantly greater than the medium control triplicate sets by Student’s t test (P < 0.05).

HPV Typing. HPV testing was conducted on lavage and ViraPap samples using the hybrid capture method formatted to detect 16 HPV types (6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, and 58), as described previously (36). Samples were screened for overall positivity, and those found to be positive were tested further using an HPV-16-specific hybrid capture probe to determine which HPV-positive samples were positive for HPV-16. For cervical tumor specimens, DNA was processed from formalin-fixed, paraffin-
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RESULTS

The characteristics of the 140 study patients are summarized according to disease status (Table 1). The age distribution of the four study groups differed, with cytological normals being youngest (median age = 28 years) and cancer patients being the oldest (median age = 42 years; P = 0.001). However, the spread of ages was not so great as to be compatible with a role for immunological senescence in influencing the results. HPV DNA data were available on 128 of the 140 subjects (91.4%). HPV DNA was detected in 32 LSIL patients (49.6%), 16 HSIL patients (76.9%), and 15 cancer patients (94%) tested. HPV-16 DNA was detected among 17 subjects with LSIL, 9 with HSIL, and 13 with invasive cancer. Among cytologically normal women with a previous history of HPV-16 infection, one (4%) was found to be currently infected with an HPV type other than HPV-16 and five (20%) were still HPV-16 positive at the time of this study due to either persistent infection or reinfection with the same virus type.

Representative results from four subjects, one from each of the four disease categories examined, are shown in Fig. 2. Subjects NL-7 (A) and LSIL-27 (B) both responded to a group of peptides at almost the same level as they responded to whole FLU, at all three dilutions. The response to peptides and FLU ranged from several-fold to 10-fold above that seen for these patients when stimulated with medium alone. Neither the response to the peptides nor that to FLU was as high as that to the mitogen, PHA. In contrast, subject HSIL-9 (C) made almost as much IL-2 in response to FLU as she did to PHA but responded only weakly, if at all, to the peptides. Subject CA-8 (D) responded well to PHA and less strongly to FLU. Such studies were performed on all subjects, but for the sake of space and clarity, responses were summarized for presentation as positive or negative for each of the groups of peptides, FLU, and PHA.

Individual response data to E6 and E7 peptides (5 μM), FLU, and PHA are presented in Table 2. There was no significant difference among the disease groups in the percentage responding to PHA, as would be expected for this mitogen (P for trend = 0.39). Because nonimmunodeficient human PBMC cultures in which the cell and culture conditions are adequate should respond to the mitogen PHA, those subjects' PBMCs that failed to produce IL-2 in response to HPV-16-specific peptides and FLU and also failed to produce IL-2 in response to PHA were considered technically inadequate, probably due to damage in shipping, and excluded from further analysis. This occurred for 11 subjects: 2 cytologically normal women, 6 women with LSIL, and 3 women with HSIL.

The proportion of subjects responding to at least one group of HPV-16 peptides declined significantly (P for trend = 0.02) with increasing severity of the disease (Table 3). When analysis was restricted to subjects positive for any HPV type or those positive for HPV-16, similar trends were observed (Table 3). Past epidemiological experience would lead one to expect a higher fraction of HPV-16-positive patients among the HSIL group than among the LSIL group (40), and this was borne out in our sample (Table 1); thus, the significant trend for responsiveness to HPV-16 peptides running in the opposite direction is even more impressive. In particular, the lack of response of 26 of 28 patients with invasive cancer cannot be explained by lack of exposure to HPV-16 because 81% of the tumors tested were HPV-16 positive.

It is important that the results do not seem to simply reflect a generalized immunodeficiency of cancer or, more particularly, an effect of the disease on the ability of T cells to make IL-2 in response to recall antigens because there was no significant difference among the four groups in the fraction of individuals responding to FLU: 47.8% of normal women, 56.7% of women with LSIL, 55.6% of women with HSIL, and 50.0% of those diagnosed with cancer were positive for IL-2 production in response to FLU (P for trend = 0.96).

Furthermore, the age of the patients was not significantly different between the IL-2 E6/E7 response-positive and -negative patients, with the mean and median age of IL-2-negative and -positive subjects being 35.7/34 years (IL-2 negative) and 36.0/35 years (IL-2 positive), respectively. Thus, the association between IL-2 response and cervical neoplasia is not confounded by the age of the patients.

When the magnitude of response to the HPV-16 E6 and E7 peptides was examined among those who responded, the levels were similar among all four groups (Fig. 3). Therefore, the significant difference between the groups that correlates with disease status is in the fraction of individuals responding, not in the magnitude of response among those who did respond.

To address the issue of which epitopes of HPV-16 E6 and E7 oncoproteins most efficiently induce IL-2 production, we summarized the data according to the group of peptides examined (Fig. 4). Among the 129 patients examined, no patient responded to E6–G1 (NH2 terminus), and the greatest number of patients responded to E6–G3 (COOH terminus). In the case of HPV-16 E7 peptides, every group could induce IL-2 response in some of the patients, although E7–G3 (COOH terminus) seemed to stimulate IL-2 production in more of the subjects than E7–G1 (NH2 terminus). Thus, for both proteins, there was a statistically nonsignificant trend to respond more to the COOH-terminal third than to the NH2-terminal third of the protein. This finding is consistent with the recent results of Kadish et al. for E7 (41). Although it was not possible with the amount of blood available to map the individual peptides within each mixture that were eliciting the responses in each subject (as we had hoped to do), we are currently trying to raise long-term T-cell lines from a small number of positive patients to map the more commonly recognized epitopes and determine HLA restriction.

DISCUSSION

The current study confirms our a priori hypothesis that Th1 cytokine production in response to HPV-16 E6 and E7 peptides is diminished in patients with HSIL and cervical cancer. In fact, although 35% of cytologically normal women with a known history of HPV-16 infection responded to HPV-16 peptides, only 17% of women with HSIL and 7% of those with invasive cancer responded to the same set of peptides. Certainly, a precedent for the role of Th1 immunity exists in other chronic diseases, such as parasitic diseases (14, 22, 42), leprosy (23), and HIV (25). In murine tumors, there is evidence that protective cellular immune responses can involve Th1-like CD4+ T cells and that CD4+ T cells that are probably Th2-like can suppress such a protective response (43-45). Also, the in vivo efficacy of CD4+ tumor-infiltrating lymphocytes in immunotherapy of cancer has been shown to be correlated to their cytokine production (46). Recently, Clerici et al. (47) have obtained data indicating a shift in cytokine response to mitogen in patients with Hodgkin’s disease, and several groups showed that Sézary cells secrete Th2-like cytokines (48, 49) and that IL-12 could convert the pattern to the Th1 type in vitro (50). However, to our knowledge, no previous study has reported an asso-

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This table summarizes IL-2 production by PBMCs stimulated with HPV peptide groups, FLU, and PHA. Results were indicated as positive (+) and negative (−) based on our criteria, as described in “Patients and Methods.”

Table 2 IL-2 response of individual subjects in response to the HPV-E6 and E7 peptides, FLU, and PHA

This column shows the categories of each subject. NL, cytologically normal, but known to have been HPV-16 DNA positive before this study; CA, invasive cancer.

All cytologically normal patients were known to have a past history of infection with HPV-16, but the results indicate the HPV positivity at the time of blood drawing. ND, not detected; NT, not tested; 16, HPV-16 positive; OTH, other HPV types positive; IM, insufficient material.

Patients had unresected tumor at the time of blood drawing.
cation between Th1 cytokine production in response to viral onco-proteins and disease stage of a human tumor.

In the present study, we measured IL-2 response to HPV-16 E6 and E7 using an in vitro assay in which a set of overlapping synthetic peptides covering most of the sequences of E6 and E7 were used to stimulate PBMCs. The overlaps were chosen to be 10 residues, so that any epitope of 11 residues or shorter would be included in at least one of the peptides. Although peptides presented by class II MHC molecules tend to be larger on average than those presented by class I MHC molecules (51), most minimal epitopes can be found within peptides of length 15, as used here, and can frequently be truncated to 11 residues or fewer with retention of activity.

Presentation of antigens to T-cells is known to be HLA-restricted (51). In the current study, the number of subjects was too small to detect any correlation between HLA haplotype and IL-2 response, and HLA typing information was obtainable on only a subset, so no analysis could be performed. However, it is unlikely that we would have detected any clear association between HLA haplotype and IL-2 response, given that we examined responses to mixtures of peptides, not single epitopes. This important issue should be addressed in future studies being planned.

The ideal study would have included measurement of Th2 cytokine responses as well as Th1 cytokine responses, and we originally planned to measure IL-4 production as well as IL-2 production. However, it has not been possible in most labs to measure human IL-4 production by bulk PBMCs to specific antigens, as opposed to mitogens (52). Even Romagnani (53) and coworkers, who first described the Th1/Th2 dichotomy in human T cell clones, have reported IL-4 production only by human T-cell clones, not bulk PBMCs. In our attempts to measure IL-4 responses to FLU and HPV peptides in the subject population of this study, we could actually detect IL-4 production in response to both FLU and HPV E6 or E7 peptides in several cases, but the number of patients was too limited to analyze (data not shown). Also, the IL-4 responses had to be measured in separate cultures from those used for IL-2 production because the anti-IL-2 receptor antibody used in the latter cultures to prevent IL-2 consumption also might interfere with IL-4 production, which has been reported to be IL-2 dependent (54). Therefore, given the limited volumes of blood available and the large number of patients in the study, we decided to focus our resources on the IL-2 response. Although we cannot address the question of whether the decreased type 1 cytokine response to HPV antigens is accompanied by an increased type 2 response, the reduced frequency of IL-2 productive response to HPV antigens stands in its own right as an indicator that a diminished cellular immune response is associated with higher grades of cervical neoplasia.

Given that the present study has a cross-sectional design, we were unable to ascertain cause and effect. One reasonable interpretation of the data is that loss of the Th1 cellular immune response to HPV antigens favors progression of the malignant disease through a lack of immune surveillance. However, the alternative interpretation is equally possible, that the progression of intraepithelial lesions causes a defective specific immune response because of either some effect of the tumor cells or an effect of the chronic viral infection itself. Although there is evidence for generalized immune defects associated with advanced cancer, it should be noted that most of the subjects in the present study had intraepithelial lesions and that the responses observed in this cross-sectional study do not seem to represent a generalized immune defect because the response to FLU was intact and not different among the four groups of subjects. Thus, if the cancer is the cause of the immune defect, it would have to be inducing a restricted, perhaps HPV antigen-specific deficit, such as peripheral tolerance.

We observed an association between IL-2 production and disease severity that was not restricted to HPV-16-positive subjects. We have no clear explanation for this lack of type specificity of the response, although several plausible possibilities exist. Subjects who responded to HPV-16 peptides but did not have detectable HPV-16 infection at

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cytopathologic criteria</th>
<th>Normal</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
<th>P*</th>
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</thead>
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<td>18</td>
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* For Mantel-Haenzel χ² test for trend.
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