
REPORTS

Stress and Immune Responses After Surgical Treatment for Regional Breast Cancer

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Background: Adults who undergo chronic stress, such as the diagnosis and surgical treatment of breast cancer, often experience adjustment difficulties and important biologic effects. This stress can affect the immune system, possibly reducing the ability of individuals with cancer to resist disease progression and metastatic spread. We examined whether stress influences cellular immune responses in patients following breast cancer diagnosis and surgery. **Methods:** We studied 116 patients recently treated surgically for invasive breast cancer. Before beginning their adjuvant therapy, all subjects completed a validated questionnaire assessing the stress of being cancer patients. A 60-mL blood sample taken from each patient was subjected to a panel of natural killer (NK) cell and T-lymphocyte assays. We then developed multiple regression models to test the contribution of psychologic stress in predicting immune function. All regression equations controlled for variables that might exert short- or long-term effects on these responses, and we also ruled out other potentially confounding variables. **Results:** We found, reproducibly between and within assays, the following: 1) Stress level significantly predicted lower NK cell lysis, 2) stress level significantly predicted diminished response of NK cells to recombinant interferon gamma, and 3) stress level significantly predicted de-

creased proliferative response of peripheral blood lymphocytes to plant lectins and to a monoclonal antibody directed against the T-cell receptor. **Conclusions:** The data show that the physiologic effects of stress inhibit cellular immune responses that are relevant to cancer prognosis, including NK cell toxicity and T-cell responses. Additional, longitudinal studies are needed to determine the duration of these effects, their health consequences, and their biologic and/or behavioral mechanisms. [J Natl Cancer Inst 1998; 90:30-6]

A diagnosis of cancer and cancer treatments are objective, negative events in an individual's life. Although negative events do not always produce stress and a lowered quality of life, data from many studies document severe, acute stress at cancer diagnosis (1) and during recovery (2). The negative psychologic responses of individuals with cancer to the diagnosis and treatment are important in their own right because these responses are targets for cancer control efforts (3,4). In addition, data suggest that stress responses are accompanied by nonrandom (i.e., correlated) negative changes in a broad range of immune responses. This study examines from a biobehavioral perspective whether stress influences cellular immunity in women with breast cancer after diagnosis of breast cancer and during the postsurgical period (5).

Meta-analyses (6,7) suggest that psychologic stress and the experience of life stressors are reliably associated with negative immune alterations in noncancer subjects; i.e., "higher" levels of stress (e.g., self-reports of stress or negative affects, such as sadness or clinical diagnoses of depression) are related quantitatively and functionally to "reduced" cellular immune responses, such as lowered natural killer (NK) cell lysis. This effect has been found regularly for individuals in the midst of chronic stressors, and some of the largest responses and

changes have been found for lengthy stressors and those that have interpersonal components.

Illustrative data come from Kiecolt-Glaser, Glaser, and colleagues (8-11), who have followed individuals during the long, stressful experience of giving care to a spouse diagnosed with Alzheimer's disease. Not surprisingly, caregivers report high levels of distress and negative affect as they cope with their relative's difficult behavior and mental deterioration (8). Moreover, these researchers have found, for example, that NK cells obtained from caregivers are less responsive to the cytokine recombinant interferon gamma (rIFN γ) and recombinant interleukin 2 (rIL-2) than are cells obtained from matched community control subjects (9). In addition, these highly stressed subjects have a poorer proliferative response to mitogens (8), exhibit substantial deficits in the antibody and virus-specific T-cell responses to an influenza virus vaccine (10), and demonstrate stress-related defects in wound repair (11).

There are fewer data on the relationship between stress and immunity among cancer patients. Levy et al. (12) reported on these relationships in 66 women with stage I or II breast cancer 3 months after treatment (lumpectomy or mastectomy with or without adjuvant therapy). In ad-

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dition to finding that estrogen receptor status predicted NK cell lysis, these researchers found that social support—a variable hypothesized to *reduce* stress—contributed significantly to a regression model predicting *higher* NK cell activity. These findings suggest that how a person responds to stress may also influence how stress, in turn, influences the immune response.

There is considerable evidence that patients with cancer express abnormal cellular immune responses; these abnormal responses have been found in patients with many different types of cancer (13–15), including breast cancer (16,17). Stressors are not generic, and they would not be expected to have identical physiologic outcomes. So too, the immune response involves a cascade of responses and events that can occur over time. For these reasons, we used a homogeneous breast cancer subject sample and timing of assessment to test the relationship between stress and several components of the cellular immune response, including NK cell and T-cell functions.

Women who had been diagnosed with breast cancer and who had undergone surgery for the breast cancer were studied before they began adjuvant therapy. Since we were interested in the contribution of stress in predicting an immune response above and beyond known correlates, we controlled for naturally occurring factors in our statistical analyses that affect the immune responses—specifically, age, disease stage (lymph node status), and recovery (days since surgery) (18). Because the immune system contains a considerable amount of redundancy, we focused on three components that would each provide important, but complementary, information.

First, we measured NK cell lysis. We chose to measure NK cell lysis because those cells are believed to act early in the immune response and they have been demonstrated to play an important role in immune surveillance against tumors and virally infected cells (19–21). Second, we measured the ability of the NK cells to respond to rIFN γ and rIL-2. It has been shown that lymphokine-activated killer (LAK) cells are highly cytotoxic against a wider variety of tumor cells than those lysed by resting NK cells (22), an effect also observed in patients with breast cancer (23). Finally, to obtain information on

the T-cell response, we measured the response of peripheral blood leukocytes (PBLs) to two mitogens—phytohemagglutinin (PHA) and concanavalin A (Con A)—and we induced proliferation by stimulating the T cells with a monoclonal antibody (MAb) to the T-cell receptor.

Subjects and Methods

Patient Eligibility and Data Collection

Participants were 116 women who had been diagnosed with invasive breast cancer and who were surgically treated within the last 4 months but who had not yet begun adjuvant treatment. Women were from 14 to 101 days (mean = 37 days; median = 33 days) after surgery for stage II (70%) or III (30%) invasive breast cancer. We used the American Joint Committee on Cancer and the International Union Against Cancer staging system. The women ranged in age from 31 to 84 years (mean = 52 years). Recruited consecutively from mid-1994 to early 1997, the majority (82%) were being treated at a National Cancer Institute-designated, university-affiliated Comprehensive Cancer Center, and the remainder (18%) were receiving treatment at local community hospitals. All women came to the General Clinical Research Center at the university where psychologic, behavioral, and medical data were collected and a 60-mL blood sample was taken from them. Assessments were conducted between 8:00 AM and 12:00 AM to reduce diurnal variability.

Stress Measure

The Impact of Event Scale (IES) (24) is a standardized self-report questionnaire used to examine intrusive thoughts (“I had dreams about being a cancer patient,” “Other things kept making me think about cancer”) and avoidant thoughts and actions (“I tried not to talk about it,” “I was aware that I still had a lot of feelings about cancer, but I didn’t deal with them”) concerning cancer. Fifteen items are used, and women rate each event or feeling in terms of the frequency of occurrence (i.e., “not at all,” “rarely,” “sometimes,” and “often”) during the previous 7 days. Scores range from 0 to 75. For this sample, descriptive statistics were as follows: range, 0–65; mean = 26; median = 25; and standard deviation = 15.2. The scale has satisfactory reliability with internal consistency of .78–.82 and a 2-week test–retest reliability of .79–.89, respectively. The validity of the measure is suggested by data indicating that individuals who experience involuntary, distress-related thoughts following traumatic life events are also those who suffer the greatest negative effects psychologically [e.g., (2)].

Immune Assays

Blood cell separation. PBLs were isolated from 60 mL of venous blood by use of Ficoll gradients (Pharmacia Biotech, Inc., Piscataway, NJ). The isolated leukocytes were then washed in calcium- and magnesium-free phosphate-buffered saline and counted on a Coulter counter (Coulter Corp., Miami, FL). Aliquots of 8×10^6 isolated PBLs were suspended again in 0.8 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.75%

sodium bicarbonate, 2 mM L-glutamine, and 10 μ g/mL of ciprofloxacin.

Quantification of total T lymphocytes, T-cell subsets, and NK cells. Isolated PBLs were absorbed with MAbs conjugated to either fluorescein isothiocyanate or rhodamine according to the cell surface marker being studied: total T cells (CD3, fluorescein isothiocyanate), T4 subset (CD4, rhodamine), T8 subset (CD8, fluorescein isothiocyanate), and NK cells (CD56, rhodamine). All MAbs were purchased from Coulter Corp. Briefly, 0.5×10^6 cells were incubated with the MAb for 15 minutes at room temperature. After the incubation, the cells were fixed, and the red blood cells were lysed with Optilyse C, a buffered solution containing 1.5% formaldehyde, according to the manufacturer’s instructions (Coulter Corp.). Samples were analyzed with the use of a Coulter EPICS Profile II flow cytometer as described previously (8).

NK cell cytotoxicity. To determine NK cell activity, a microtiter ^{51}Cr -release cytotoxicity assay was used as described previously (9,25). The target cells used were K-562 cells, an NK cell-sensitive human myeloid cell line. Target cells, labeled overnight for 16 hours with ^{51}Cr , were placed in triplicate wells of 96-well V-bottom plates, and PBLs were added, resulting in effector-to-target (E:T) cell ratios of 100:1, 50:1, 25:1, 12.5:1, and 6.25:1.

NK cell response to cytokines. Procedures for treatment of PBLs with rIFN γ and rIL-2 involved preparing isolated PBLs at a concentration of 3×10^6 cells/mL in complete RPMI-1640 medium and then seeding the cells into three replicate tissue culture tubes (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) at 6×10^6 cells per tube. Cells were incubated in complete RPMI-1640 medium alone or complete medium supplemented with 250 IU/mL rIFN γ or 60 IU/mL rIL-2 (Genzyme, Boston, MA). Cell suspensions were gently mixed and then incubated at 37 °C in an atmosphere of 5% CO₂ for 65 hours. For the assay, triplicate aliquots of cell suspensions were placed in wells of V-bottom plates, with E:T cell ratios of 50:1, 25:1, 12.5:1, 6.25:1, or 3.13:1. In addition, six wells with target cells and medium only and target cells with detergent (5% sodium dodecyl sulfate in phosphate-buffered saline) were prepared to determine spontaneously released chromium and maximal lysis, respectively. The plates were centrifuged at 300g for 5 minutes at 20 °C to bring the effector and target cells into close contact; they were then incubated at 37 °C in an atmosphere of 5% CO₂ for 5 hours. After this incubation, the plates were centrifuged at 300g for 5 minutes at 20 °C, 100 μ L of supernatant was collected from each well, and counts per minute were determined by use of a Beckman 9000 gamma counter (Beckman Instruments, Inc., Fullerton, CA) as described previously (9,26).

Blastogenic response to PHA, Con A, and MAb to the T3 receptor. The concentrations for PHA and Con A used were 2.5, 5.0, and 10.0 μ g/mL. To measure the blastogenic response to the MAb to the T-cell receptor, we used the following three dilutions of the purified MAb: 32:1, 64:1, and 128:1. For all three assays isolated, PBLs seeded in triplicate at 0.5×10^5 per well were incubated for 68 hours at 37 °C in 96-well flat-bottomed plates and then labeled for 4 hours with MTS, i.e., 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

(Promega Corp., Madison, WI) to measure proliferative response. Briefly, the MTS procedure is a nonradioactive calorimetric procedure that labels metabolically active cells via reduction of a colored substrate. The amount of proliferation was determined by optical density of the suspension in the well. Optical density determinations were performed by use of a Titertek Multiscan MCC microplate reader (Flow Laboratories, Inc., Finland) at a determination wavelength of 492 nm and a reference wavelength of 690 nm as has been noted (27,28).

Statistical Analyses

Preliminary analyses. Before conducting the principal analyses, we checked the data for the contribution of "nuisance" variables (covariates) that could potentially be related to psychologic stress, immune outcomes, or both [see (25) for a discussion]. The variables examined were measures of aspirin, alcohol, caffeine, and nicotine intake; amount of sleep; plasma albumin level (as an indicator of nutritional status); incidence of recent infectious illness; and the Karnofsky performance status rating. We examined the relationships between these variables and each of the three sets of outcome variables: NK cell lysis, ability of NK cells to respond to rIFN γ and rIL-2, and the blastogenic response of PBLs to Con A, PHA, and the T3 MAb. Analysis of variance was used for the categorical independent variables, and simple correlations were used for numerically scaled independent variables.

Screening of these potential covariates involved examination of the relationships between 11 covariates and 20 dependent variables, or a total of 220 bivariate associations. Of these 220 associations, 15 were found to be statistically significant at .05 significance level. This number of significant effects is only slightly more than would be expected by chance alone (i.e., $220 \times .05 = 11$). Inspection of the significant relationships showed that many of them were attributable to the influence of a few outliers in the data. To be conservative, all of the regression analyses described below were run twice, once including and once excluding those covariates that had significant bivariate associations with the relevant dependent variables. In no case were results of the regression analyses significantly altered by the inclusion of the covariates. Given this fact and the consistently weak relationships of the covariates to the dependent variables, we do not report further results involving the covariates.

Principal analyses. The principal analyses assess the relationship between the IES measure of psychologic stress and the following three sets of outcome measures: 1) NK cell lysis at five E:T ratios, 2) response of NK cells to rIFN γ and rIL-2 stimulation at five E:T ratios each, and 3) the PBL blastogenic response to PHA and Con A and proliferative response to the T3 MAb at three concentrations or dilutions each.

We were interested in the role of stress in predicting these outcomes, over and above the impact of disease and recovery variables on the immune response. Thus, we chose to control for three variables: 1) age, which is associated with down-regulation of the immune system; 2) disease stage, which is an indicator of the extent or burden of disease; and 3) days since surgery, which is an indicator of the degree of recovery from surgical stress and related factors (e.g., anesthesia).

Using hierarchical multiple regression (29), we tested the predictive value of psychologic stress for the measured immune outcomes. This procedure enters variables in a specified sequence and, at the final step, provides a test of the variance of the dependent variable (immune outcome) due to the predictor (stress), above and beyond the contribution of the control variables (age, stage, and days since surgery). In these regression analyses, age, days since surgery, and IES were considered as numerical variables. Stage was a categorical variable with two levels: II versus III.

For all of the analyses described below, any missing data were managed by the pairwise deletion technique, wherein each bivariate association is estimated with the use of all subjects for whom measures on both variables are available. This approach allows for more complete usage of available data than do alternative procedures (e.g., listwise deletion). For all of the dependent variables except the response of NK cells to rIFN γ , the quantity of missing data was small—with never more than 10 observations missing for any bivariate association. Effective sample sizes for the regression analyses ranged from 113 for the NK cell lysis ratios to 103 for T3 MAb values. For rIFN γ measures, sample sizes varied from 85 to 49 across the range of concentrations employed.

For each analysis, we provided three regression models: models A, B, and C. Model A includes only the control (independent) variables (i.e., age, stage, and days since surgery) in predicting the immune outcome (e.g., NK cell lysis). Predictors in model A were introduced simultaneously because we had

no basis for or a strong interest in investigating their effects in any particular sequence. Model B includes the three control variables as well as the psychologic stress variable (IES) in the prediction of the immune outcome. Of particular interest in this analysis was the increment in the squared multiple correlation (R^2) from model A to model B (i.e., R^2_{B-A}), indicating variance in a dependent variable (e.g., NK cell lysis) attributable to stress (IES) beyond that explained by the control predictors. In addition, the standardized regression beta (β) for the psychologic stress variable (IES) in model B (i.e., β_{Stress}) indicates the magnitude and direction of the influence of this predictor on the dependent variable. The significance of the β weight was also tested. Finally, model C indicates the contribution of psychologic stress as the lone predictor; this third model provides the simple association between psychologic stress and immune function.

Results

Analyses Predicting NK Cell Lysis

Table 1 provides the results from the three models, A, B, and C, predicting NK cell lysis. For model A, in which age, stage, and days since surgery are the independent variables, R^2_A was small and nonsignificant for every E:T ratio (all F ratios were <1.0). Because the percentage of NK cells available would influence the

Table 1. Results of regression analyses for predicting natural killer (NK) cell lysis across six effector-to-target cell (E:T) ratios

	Dependent variable: NK cell lysis at E:T ratios					
	100:1	50:1	25:1	12.5:1	6.25:1	3.125:1
Model A, R^2_A *	.005	.007	.012	.015	.020	.023
Model AA, R^2_{AA} †	.085	.148	.185	.233	.250	.241
Model B ‡						
R^2_B	.135	.212	.238	.268	.275	.253
R^2_{B-AA} §	.050	.064	.053	.035	.025	.012
β_{Stress}	-.234	-.265	-.240	-.194	-.165	-.115
$t(df = 110)$ ¶	-2.462	-2.921	-2.672	-2.223	-1.892	-1.280
P	.016	.004	.008	.028	.062	.204
Model C #						
R^2_C	.067	.091	.084	.066	.056	.032
$t(df = 110)$ ¶	-2.826	-3.338	-3.199	-2.811	-2.558	-1.867
P	.006	.002	.002	.006	.012	.066

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, NK cell lysis. The R^2_A is the total variance in NK cell lysis explained by these three predictors.

†Model AA includes model A variables plus the control predictor percentage of NK cells for the immune outcome, NK cell lysis. The R^2_{AA} is the total variance in NK cell lysis explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, NK cell lysis. The R^2_B is the total variance in NK cell lysis explained by the four control predictors and the stress predictor.

§ R^2_{B-AA} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the NK cell lysis outcome.

|| β_{Stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶ df refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, NK cell lysis. The R^2_C is the total variance in NK cell lysis explained by stress; this model provides the simple association between psychologic stress and immune function.

total NK cell activity as measured by lysis, we next added the percentage of NK cells, as determined by flow cytometry, into the analyses as an additional, independent control variable as shown (model AA). Across all E:T ratios, the R^2_{AA} values suggested that this variable added significant variance, as predicted, yielding R^2_{AA} values ranging from .085 to .250.

More important was the addition of the stress variable (IES) as a predictor, shown in model B. The value of R^2_B for lysis was noticeably larger than that of R^2_{AA} , and it provided a significant increment in prediction across the E:T ratios. These data indicate that the measure of psychological stress that was used accounted for significant variance in NK cell lysis above and beyond that explained by age, stage, days since surgery, and percentage of NK cells. Moreover, the sign of the β regression coefficient for IES was negative, as predicted, indicating that an increase in measured stress was associated with a decline in NK cell lysis. The t tests for these coefficients were significant at five of the six E:T ratios. Also, no other predictor in model B had a significant regression coefficient.

We also provide the regression results when only IES was used as a predictor, eliminating the control predictors from the model (model C in Table 1). These results showed that the simple association between IES and NK cell lysis was statistically significant at five of the six E:T ratios.

Analyses Predicting Response of NK Cells to Cytokines

Results for the NK cell response to rIFN γ are provided in Table 2 and show a similar pattern. For model A, which used age, stage, and days since surgery as the independent variables, the value of R^2_A was small to moderate, ranging from .025 to .138. When stress (IES) was added to the model B regression, the R^2 values were statistically significant at all but one E:T ratio (50:1). Furthermore, the increments in the prediction due to IES, R^2_{B-A} , were significant and ranged from .054 to .119. This value reflects the proportion of variance in the cell response accounted for by stress (IES) beyond that explained by the control variables. Again, the negative weight of β for IES in model B indicated a negative influence of psychological stress on the response of the NK

Table 2. Results of regression analyses for predicting natural killer (NK) cell response to recombinant interferon gamma (rIFN γ) across five effector-to-target cell (E:T) ratios

	Dependent variable: NK cell response to rIFN γ at E:T ratios				
	50:1	25:1	12.5:1	6.25:1	3.125:1
Model A, R^2_A *	.025	.097	.080	.138	.124
Model B†					
R^2_B	.041	.151	.197	.257	.208
R^2_{B-A} ‡	.016	.054	.117	.119	.084
β_{Stress} §	-.128	-.244	-.358	-.358	-.301
t	-1.104	-2.190	-3.203	-3.084	-2.083
df	82	81	74	65	46
P	.274	.032	.002	.004	.044
Model C¶					
R^2_C	.015	.077	.149	.149	.088
t	-1.128	-2.586	-3.581	-3.343	-2.080
df	82	81	74	65	46
P	.264	.012	.002	.002	.044

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, NK cell response. The R^2_A is the total variance in NK cell response explained by these three predictors.

†Model B includes model A control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, NK cell response. The R^2_B is the total variance in NK cell response explained by the three control predictors and the stress predictor.

‡ R^2_{B-A} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the NK cell response.

§ β_{Stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

|| df refers to the degrees of freedom in model B.

¶Model C includes stress as the only predictor of the immune outcome, NK cell response. The R^2_C is the total variance in NK cell response explained by stress; this model provides the simple association between psychological stress and immune function.

cells to rIFN γ . Again, no other predictor in model B had a significant regression coefficient. Finally, the results for model C in Table 2 showed a simple association between IES and the rIFN γ response. These correlations were significant at four of the five E:T ratios; the proportions of variance accounted for were in the range of .077 to .149.

We attempted to calculate a parallel set of regressions for the response of NK cells to rIL-2. However, cells from a large proportion of the patients (62%) had no response to rIL-2. When the regressions were conducted on data obtained from the remaining patients (38%), the addition of stress (IES) in model B produced a significant R^2 value at the 25:1 E:T ratio only. It appeared that the majority of the subjects' NK cells did not respond to treatment with rIL-2.

Analyses Predicting Blastogenic Response of PBLs to Con A, PHA, and the T3 MAb

Table 3 shows regression results for the Con A and PHA blastogenic responses across three concentrations each. Because the findings are similar for both assays, they will be discussed together.

For model A, which used age, stage, and days since surgery as the independent variables, the value of R^2_A for Con A ranged from .035 to .054 and was of similar magnitude for PHA, ranging from .022 to .033. Since the number of total T cells available will affect the blastogenesis values, we next added the number of T3-positive cells into the analyses as an additional, independent control variable as shown by the step model AA. Across all concentrations for each mitogen, the value of R^2_{AA} suggested that this variable added variance, yielding the R^2_{AA} values ranging from .105 to .125 for Con A and from .023 to .033 for PHA.

The addition of stress (IES) to the regression for blastogenesis added significant variance, as indicated in model B. All of the R^2 values were statistically significant. Considering the increments in R^2 due to stress (IES), these were significant and ranged from .032 to .061 for Con A and from .047 to .060 for PHA, reflecting the proportion of variance in the blastogenesis accounted for by IES beyond that explained by the control variables. Again, the negative β weights for IES in model B indicated a negative influence of psychological stress on the blastogenic responses

Table 3. Results of regression analyses for predicting the blastogenic response to concanavalin A (Con A) and phytohemagglutinin A (PHA) across three concentrations each

	Dependent variable: blastogenic response of mitogen					
	Con A			PHA		
	10 μg/mL	5 μg/mL	2.5 μg/mL	10 μg/mL	5 μg/mL	2.5 μg/mL
Model A, R^2_A *	.035	.043	.054	.022	.024	.033
Model AA, R^2_{AA} †	.105	.125	.115	.023	.024	.033
Model B‡						
R^2_B	.166	.174	.147	.083	.074	.080
R^2_{B-AA} §	.061	.049	.032	.060	.050	.047
β_{Stress}	-.255	-.229	-.187	-.256	-.234	-.229
$t(df = 103)$ ¶	-2.668	-2.401	-1.927	-2.521	-2.299	-2.254
P	.010	.018	.058	.014	.024	.026
Model C#						
R^2_C	.053	.065	.053	.070	.054	.052
$t(df = 108)$ ¶	-2.443	-2.724	-2.443	-2.857	-2.489	-2.441
P	.016	.008	.016	.006	.014	.016

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, blastogenesis. The R^2_A is the total variance in blastogenesis explained by these three predictors.

†Model AA includes model A variables plus the control predictor of number of T cells for the immune outcome, blastogenesis. The R^2_{AA} is the total variance in blastogenesis explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, blastogenesis. The R^2_B is the total variance in blastogenesis explained by the four control predictors and the stress predictor.

§ R^2_{B-AA} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the blastogenesis outcome.

|| β_{Stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶ df refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, blastogenesis. The R^2_C is the total variance in blastogenesis explained by stress; this model provides the simple association between psychologic stress and immune function.

across concentrations. Moreover, no other predictor in model B had a significant regression coefficient. Finally, results for model C in Table 3 showed a simple association between stress (IES) and the blastogenic response. These correlations were significant for each concentration of Con A and PHA.

Table 4 shows regression results for the proliferative response of T cells to three different dilutions of the T3 MAb. For model A, the control R^2 values were not significant for any dilution. Addition of number of T3-positive cells available as a control increased the variance accounted for as shown by the step model AA. The R^2_{AA} values ranged from .088 to .143. However, increments in R^2 due to the addition of stress (IES), as shown by R^2_{B-AA} , were significant, ranging from .056 to .067. This indicates that about 6% of the variance was accounted for by stress (IES) beyond that explained by the control variables. Once again, no other predictor in model B had a significant regression coefficient. Results for model C again showed the simple, significant as-

sociation of stress (IES) with the response to the T3 MAb at all dilutions, with R^2_C values of .092 to .102.

Discussion

Any immune response involves a complex cascade of events that occur over time. Studies suggest that the peripheral products of stress can play numerous roles in regulating immunity, and so the effects of stress will, necessarily, be variable. Current research suggests, for example, that the acute stressors, both real stressors [e.g., parachute jumps (30)] and artificial stressors [e.g., experimental tasks including speech or math stress (31)], are correlated with the mobilization (increase) of NK cells. These changes are thought to be a result of alterations in cell trafficking. In contrast, studies of chronic stressors [e.g., bereavement, caregiving, or divorce (7,9)] suggest that stress can have an effect on the ability of NK cells to lyse a target cell, the ability of NK cells to respond to rIFN γ and rIL-2 *in vitro*, and other aspects of the cellular immune response.

Our results suggest that stress, as assessed via a self-report measure of intrusive and avoidant thoughts and behaviors about cancer, was related to a negative effect on NK cell lysis, the ability of NK cells to respond to two cytokines, the blastogenic response of PBLs to two mitogens, and the proliferative response to MAb T-cell receptor. These effects were inhibitory and of similar magnitude (i.e., reliable), both between the assays and within an assay (i.e., across E:T ratios and mitogen concentrations). The analyses controlled for variables that might also be expected to exert short-term or long-term effects on immunity—such as age, stage of disease, and days since surgery—and ruled out other potentially confounding variables (e.g., nutritional status) that might also be influential. These controls reduced the plausibility of alternative, rival hypotheses for these consistent findings.

It is recognized that NK cells mediate natural immunity, but some researchers (32) suggest that their role in health generally has been underestimated. For example, there is evidence to suggest that the NK cells participate either directly or indirectly in multiple developmental, regulatory, and communication networks of the immune system. Furthermore, NK cells are efficient effector cells that not only are equipped for cell killing, but also are capable of rapid responses to exogenous or endogenous signals by producing cytokines and other factors involved in interactions between immune and non-immune cells (20).

The ability to spontaneously lyse a broad range of infected cells or tumor cells is the best known functional attribute of NK cells (20,22). Consistent with previous reports, these data suggest that stress may impair this important process. Our findings highlight the specific effect of cancer stress on immune function, whereas prior data obtained by Levy et al. (33) had suggested that women's reports of fatigue were related to lower levels of NK cell lysis. Chronically low levels of NK cell activity occur in patients with cancer, particularly when there are large tumor burdens or disseminated metastases (32). In general, patients with low NK cell activity appear to be at higher risk for infections, to have more prolonged diseases, or to suffer more severe symptoms

Table 4. Results of regression analyses for predicting proliferative response of peripheral blood leukocytes to a monoclonal antibody to T-cell receptor (T3) across three dilutions

	Dependent variable: proliferative response at dilutions		
	128:1	64:1	32:1
Model A, R^2_A *	.026	.052	.064
Model AA, R^2_{AA} †	.088	.104	.143
Model B‡			
R^2_B	.155	.160	.200
R^2_{B-AA} §	.067	.056	.057
β_{Stress} ¶	-.273	-.249	-.252
$t(df = 101)$ ¶¶	-2.747	-2.514	-2.604
P	.008	.014	.012
Model C#			
R^2_C	.102	.092	.094
$t(df = 101)$ ¶¶	-3.452	-3.255	-3.307
P	.002	.002	.002

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, proliferative response. The R^2_A is the total variance in proliferation explained by these three predictors.

†Model AA includes model A variables plus the control predictor of number of T cells for the immune outcome, proliferation. The R^2_{AA} is the total variance in proliferation explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, proliferation. The R^2_B is the total variance in proliferation explained by the four control predictors and the stress predictor.

§ R^2_{B-AA} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the proliferation outcome.

¶ β_{Stress} is the standardized beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶¶ df refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, proliferation. The R^2_C is the total variance in proliferation explained by stress; this model provides the simple association between psychologic stress and immune function.

than patients whose NK cell activity remains normal (32,34).

A variety of biologic response modifiers are known to increase the activation, proliferation, or cytotoxicity of NK cells (20). Among the best known activators of NK cells are IL-2 and IFN γ . Our data show that the physiologic changes associated with psychologic stress inhibited NK cell lysis. Stress also affected the ability of NK cells to respond to rIFN γ , a finding that is consistent with two previous reports involving another life stressor [i.e., caregiving for a spouse with Alzheimer's disease (9,26)]. It is interesting that NK cells from 62% of the women did not respond to rIL-2. In subsequent analyses comparing women who did have an rIL-2 response with those who did not, no stress or disease variable differentiated the two groups. Further studies will need to be performed to explore this result, although it is possible that the lack of responsiveness of NK cells to rIL-2 may be due to an overproduction of prostaglandin E_2 by monocytes. It has been suggested that in breast cancer patients prostaglandin E_2 decreases IL-2 production in effector cell populations, resulting in the down-

regulation of the expression of the IL-2 receptor on NK cells (23). Follow-up studies will need to pursue and clarify this difference in cytokine responses.

It has been shown that the ability of PBLs to respond to PHA is reduced, in general, in cancer patients (35); this lowered response is related to tumor burden and declines in the ability of PBLs to respond to PHA with disease progression (36). The negative effect of stress on blastogenesis was replicated in this study across two mitogens, PHA and Con A, as well as in the response of T cells to an MAb against the T-cell receptor. These findings are consistent with correlational and experimental studies indicating that stress impairs the blastogenic response of PBLs to mitogens and virus-specific T-cell responses (8,10,37-39). Mitogen-induced proliferation has been used to indicate the immune system's ability to respond to antigens from pathogens. Chronically stressed, but healthy, individuals showing decrements in the cellular immune response (including NK cell lysis and the response of the PBLs to mitogens) subsequently reported a higher incidence of infectious illnesses (8). If this

effect is reliable, these data would suggest that cancer patients who experience high levels of stress, lowered levels of responsive T lymphocytes, and decreased NK cell function may be at greater risk for infectious illnesses as they begin adjuvant therapy.

It is interesting that evidence is accumulating to suggest that psychologic and/or behavioral stress reduction interventions may enhance certain aspects of the cellular immune response, including NK cell lysis. In an early investigation, Kiecolt-Glaser et al. (40) studied 61 healthy adults living in a retirement home. After receiving 1 month of training in progressive muscle relaxation, the subjects showed evidence of a 30% increase in NK cell lysis in comparison with those who received no treatment or only social contact. Fawzy et al. (41) studied 61 patients with melanoma and reported that, 6 months after treatment, subjects receiving intervention had significantly higher levels of IFN alfa-augmented NK cell activity than those who received no treatment. These data suggest that, if behavioral interventions can reduce stress and enhance the cellular immune response, then health outcomes might improve.

In conclusion, these data show a down-regulation of different aspects of the cellular immune response associated with the psychologic stress that accompanies the diagnosis and initial surgical treatment of cancer. We note that these study participants are part of a larger effort testing the biobehavioral aspects of stress, immunity, and disease course (5). It will be important to document the longitudinal nature of these findings, and future studies will provide such data. Moreover, half of the women who participated have been randomly assigned to receive a psychologic/behavioral intervention specifically designed to reduce stress, enhance quality of life, and test for the biologic mechanism—such as immune responses—that may mediate any positive effects of stress reduction on health and disease outcomes.

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Notes

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Estrogen Receptor Expression in Benign Breast Epithelium and Breast Cancer Risk

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Background: Estrogen exposure is a major risk factor for breast cancer. Increased estrogen responsiveness of breast epithelium may enhance this effect. We examined the relationship between breast cancer diagnosis and 1) the presence and absence of estrogen receptor expression in benign breast epithelium, 2) the level of expression and 3) its variation during the menstrual cycle, and 4) other established risk factors. e.g., age, age at menarche, parity, and family history. **Methods:** We measured estrogen receptor expression (as % of positive cells) by immunohistochemistry in normal breast epithelium from 376 women undergoing diagnostic or therapeutic breast surgery. Data on established risk factors were collected prior to surgery and those on menstrual cycle dates at the time of surgery. Logistic regression was used to assess risks (odds ratios [ORs]). **Results:** The crude OR for breast cancer in women with estrogen receptor-positive breast epithelium versus those without was 3.16 (95% confidence interval [CI] = 1.89–5.28), with an OR of 2.49 (95% CI = 1.25–4.96) for premenopausal and an OR of 3.32 (95% CI = 1.43–7.68) for postmenopausal women. The ORs remained high and statistically significant after controlling for age and other breast cancer risk factors. The level of estrogen receptor expression was higher in patients with breast cancer than in control subjects and it was related to breast cancer risk in postmenopausal women (P trend <.005). Expression declined as expected in premenopausal control subjects as the menstrual cycle progressed but rose in breast cancer patients (P trend <.015). **Conclusions:** The overexpression of estrogen receptors in normal

breast epithelium may augment estrogen sensitivity and hence the risk of breast cancer. [J Natl Cancer Inst 1997;89:37–42]

Estrogen exposure is a major contributor to the risk of developing breast cancer, but the biologic mechanisms involved are poorly understood. One of the links is probably through the induction of proliferation of breast epithelium because every mitotic event provides an opportunity for genetic mishaps (1–3). Other possible mechanisms include a genotoxic role for estrogen metabolites (4) and high levels of both total and free estradiol in the sera of breast cancer case subjects compared with those of control subjects (5–7). Enhanced estrogen responsiveness of the target organ (breast epithelium) may be partly responsible for breast cancer susceptibility but has not been systematically explored. This forms the basis of this study.

Estrogen response requires transport of estrogen into the cell, binding of estrogen to estrogen receptors, binding to DNA, and transcription of estrogen-responsive genes (8), one of which is the gene for progesterone receptor (9,10). Given the obligate role of estrogen receptor in estrogen response and the fact that steroid receptor content appears to limit cellular response to steroids (11,12), we hypothesized that the histologically normal breast epithelium of women with breast cancer (case subjects) may demonstrate an increased estrogen receptor content when compared with women with benign breast disease (control subjects). We have previously reported data on 120 women, which show a significantly greater prevalence of estrogen receptor-positive epithelium in case subjects over control subjects (85% versus 55%) (13). We next hypothesized that estrogen receptor alpha content of breast epithelium may be determined by factors, such as age at menarche, parity, and menopause, or it may be influenced by use of exogenous estrogens. We now report data on a total of 376 women with an examination of the relationship of estrogen receptor positivity to other risk factors that are endocrine related, e.g., differences in thresholds for estrogen receptor positivity, and of estrogen receptor variation during the menstrual cycle.

Methods

Our study subjects were recruited from the Breast Care Center at University Hospital, Syracuse, NY; the study was approved by the Institutional Review Board. All patients completed a self-administered questionnaire regarding breast cancer risk factors at their first clinic visit. For most women, surgery was performed within 1 month of questionnaire completion. If the interval between questionnaire completion and study participation exceeded 1 year, the questionnaire information was updated by direct questioning. Potential study subjects were asked to participate by allowing use of a sample of their normal breast tissue (i.e., tissue of normal gross appearance) from the surgical specimen. All participants signed a document of informed consent. Case subjects were women with newly or previously diagnosed invasive or *in situ* breast cancer who required further breast surgery, and control subjects were women without a prior history of breast cancer who required diagnostic breast biopsy, but proved not to have breast cancer. Seven cases were previously diagnosed with breast cancer; the interval from cancer diagnosis to the biopsy that resulted in study participation ranged from 7 months to 3 years. The indication for biopsy in six of these women was the appearance of a new breast lump or mammographic density in the contralateral breast. One woman underwent a prophylactic contralateral mastectomy 3 years after the diagnosis of her original duct carcinoma *in situ*. Indications for diagnostic biopsy included palpable lumps, mammographic abnormalities, and nipple discharge. Benign lesions encountered in these biopsy specimens ranged from fibroadenoma to various types of nonproliferative fibrocystic disease and proliferation without atypia, including sclerosing adenosis. Women with atypical hyperplasia on biopsy were not specifically excluded from being control subjects, but in fact only one control subject was found to display atypical proliferation in her biopsy specimen.

The study population was accrued between December 1990 and December 1995. Fresh surgical specimens were reviewed by a pathologist, who released a grossly normal sample of breast tissue for study if he/she felt that making an accurate diagnosis of the patient's condition would be unaffected by permitting the use of this sample. The potential study population comprised 1620 women undergoing breast surgery at University Hospital during this interval. Breast tissue was released for this study by the examining pathologist on 531 (32.8%) of these women, who formed the actual study population. The decision not to release tissue for research was based solely on specimen size and the need to evaluate surgical margins or exclude a diagnosis of carcinoma *in situ*. The mean age of the actual study

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population was 46.8 years as opposed to 45.1 years in the potential population; menarche prior to age 12 years occurred in similar proportions of the actual and potential populations (146 [27.5%] of 531 versus 421 [26.0%] of 1620); menopause after the age of 50 years occurred in 72 (13.6%) of 531 of the actual as opposed to 179 (11.0%) of 1620 in the potential study population. Of the potential study subjects, 300 (18.5%) of 1620 reported a positive family history of breast cancer, whereas of the actual study population, 121 (22.8) of 531 did so.

If the surgical procedure was a mastectomy, four samples were obtained, one from each quadrant. The samples were embedded in tissue-freezing medium (O.C.T., Miles Chemical Co., Elkhart, IN) and snap-frozen in liquid nitrogen. Cryostat sections were first evaluated by hematoxylin–eosin staining. The sections were processed further only if adequate normal epithelium was present. Adequacy was defined as a minimum of 10 ducts or lobular acini. For mastectomy specimens, the sample demonstrating the best normal epithelium on hematoxylin–eosin staining was chosen for immunohistochemical demonstration of receptor expression. Epithelial samples included in the study showed either morphologically normal epithelium or minimal nonproliferative benign change. The tissue was adequate for evaluation of receptor status in 398 women; 22 subjects were excluded for incomplete information on risk factors, leaving a final study population of 376 women. Of these, 219 were premenopausal (70 case subjects and 149 control subjects) and 157 were postmenopausal (104 case subjects and 53 control subjects). Exclusions for inadequate epithelium were mainly in postmenopausal control subjects whose samples consisted of fatty tissue with very scant epithelium.

Immunohistochemistry

Five-micron cryostat sections were fixed in 10% formaldehyde, methanol, and acetone, according to the manufacturer's instructions for the estrogen receptor–immunocytochemical assay and progesterone receptor–immunocytochemical assay kits (Abbott, Chicago, IL); primary antibodies were applied (antiestrogen receptor antibody H222 and anti-progesterone receptor antibody KD68, respectively) for 30 minutes and treated with bridging antibody (goat anti-rat). PAP (peroxidase/antiperoxidase) complex was added for 30 minutes followed by a phosphate-buffered saline wash and chromagen (diaminobenzidine). The dilution of reagents used was in accordance with the manufacturer's directions. A hematoxylin counterstain was used. Proliferative epithelium was excluded from analysis; only normal epithelium and tissue with mild degrees of adenosis (mild architectural distortion without hyperplasia) were analyzed. Sections were scored positive or negative for receptor expression based on counts of an average of 2000 cells at $\times 40$ magnification. On the average, every third epithelium-containing field was counted. Labeling index was calculated as the percent of immunostained epithelial nuclei. The method of scoring benign tissue was developed in our laboratory and has been internally validated as having an interobserver variation of less than 20%. The threshold for positivity for both estrogen receptor and progesterone receptor was prospectively set at an labeling index of 1%. This threshold was chosen because it is equivalent to a level of 10 fm/mg cytosol protein in ligand-binding assays (14). Nega-

tive control slides were processed for every patient in a similar manner.

Statistical Methods

The primary relation of interest was that of estrogen receptor positivity with the occurrence of breast cancer. Twenty-two subjects were excluded at the outset for incomplete risk factor information because their risk factor questionnaires were missing. Women with missing information on one or two parameters were included in the calculation of crude and age-adjusted ORs but were excluded from the multivariate analysis reported in Table 1. Since prior studies have indicated differences in breast cancer risk factors in premenopausal and postmenopausal women, the results were stratified by menopausal status. Menopausal status was determined from the self-administered questionnaire according to patient response. Women who indicated that they were postmenopausal were considered to be so. Women who had undergone hysterectomy without oophorectomy were considered postmenopausal. Only three of these women were less than 50 years old. Analysis of the data involved a three-stage process. A univariable analysis was conducted first to determine the distributions of the study variables. This was followed by a bivariable analysis for investigation of possible confounders, outliers, and collinearity. Covariates in the analyses were defined as age at breast tissue sampling, age at menarche, parity, use of synthetic hormones (hormone-replacement therapy [HRT] or birth control pills), body mass index (kg/m^2), five categories of alcohol use (never, less than once a month, one to eight times per month, three to six times per week, and more than six times per week), previous diagnosis of cancer at any site, history of breast cancer in mother and/or sisters, prior exposure to radiation therapy, ethnicity, history of breast-feeding, prior hysterectomy and/or oophorectomy, smoking history, marital status, and educational level. In the adjusted analyses, only present oral contraceptive and HRT use was considered. Thus, a postmenopausal woman who was currently using HRT, but had used oral contraceptives 20 years previously, was considered an HRT user. Some information was missing on selected covariates, since not every woman who had a benign tissue sample completed all items on the questionnaire. These were coded as missing and were treated as such in the final analysis.

An additional variable was generated to indicate the nulliparous interval, i.e., the number of years between menarche and first-term pregnancy in parous women and menarche and menopause in nulliparous women. This variable reflects the period of time that the breast epithelium was exposed to ovarian hormones, without the effect of a term pregnancy. We developed the nulliparous interval parameter as a biologically sound way of increasing the power of the interaction analyses. The nulliparous interval was included in the analysis as a continuous variable.

Univariable and bivariable analyses were performed using SYSTAT (SYSTAT Inc., Evanston, IL). Initial comparisons of the distribution of estrogen and progesterone receptor-labeling indices were performed using the Wilcoxon rank sum test.

Multiple logistic regression analysis was used to investigate possible effect modification and to adjust for confounding (15). Receptor positivity was set at

1% or more of epithelial cells demonstrating nuclear stain. When investigating the effect of increasing positivity with risk, the categories were set arbitrarily at less than 1%, 1.00–4.99; and 5% or greater; for tests of trend, these categories were coded 0, 1, and 2. Unconditional logistic regression was performed using EGRET (Statistics and Epidemiology Research Corporation, Seattle, WA). Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for receptor positivity were calculated. The likelihood ratio test was used to assess the significance of variables in the model.

To analyze the effect of menstrual cycle on estrogen receptor expression, data were collected on premenopausal women regarding the day in the menstrual cycle when the tissue biopsy was performed. Day 1 in the cycle was the first day of menstruation. The menstrual cycle was *a priori* divided into six phases based on known differences in plasma concentrations of estradiol and progesterone throughout the cycle. These were days 1–5, 6–9, 10–14, 15–18, 19–24, and 25–28. The length of cycle for each woman was normalized to a 28-day cycle, maintaining a constant 14-day luteal phase. Median differences of estrogen receptor positivity were investigated for case subjects and control subjects, and ORs across the six phases were calculated.

Results

Case subjects and control subjects had similar demographic characteristics, except that case subjects were significantly older than control subjects (mean age, 54 versus 41 years). The proportion of case subjects and control subjects with a positive family history (i.e., an affected first-degree relative) was similar (22.4% and 22.3%); 21% of the case subjects experienced menarche prior to the age of 12 years versus 22% of the control subjects; parity before the age of 30 years occurred in 17% of the case subjects and in 11% of the control subjects; and 24% of the case subjects experienced menopause after the age of 50 years as opposed to 15% of the control subjects. None of these differences were statistically significant.

Receptor Positivity in Case Subjects and Control Subjects

Estrogen receptor and progesterone receptor positivity were examined initially as continuous variables and both were found to be positively skewed. The median estrogen receptor labeling index was 7.7 in the case subjects and 3.2 in the control subjects ($P = .001$). The median progesterone receptor labeling index was 12.4 in the case subjects and 14.6 in the control subjects ($P = .829$). Estrogen re-

ceptor positivity (threshold labeling index, 1%), examined as a categorical variable by menopausal status, was significantly different between case subjects and control subjects. In premenopausal women, there was a 17.6% difference in the prevalence of estrogen receptor positivity in benign breast epithelium, with 81.4% (57 of 70) of the case subjects being estrogen receptor positive versus 63.8% (95 of 149) of the control subjects (chi-squared test, $P < .008$). Among postmenopausal women, the difference was 18.7% (88.5% [92 of 104]) in the case subjects and 69.8% (37 of 53) in the control subjects ($P < .004$).

The crude ORs for breast cancer were elevated in women showing at least 1% breast epithelial cells positive for estrogen receptor: among all estrogen receptor-positive women, it was 3.16 (95% CI = 1.89–5.28). When stratified by menopausal status, the crude OR for premenopausal women was 2.49 (95% CI = 1.25–4.96) and for postmenopausal women was 3.32 (95% CI = 1.43–7.68). There was no significant difference in the relation between estrogen receptor positivity and breast cancer for premenopausal and postmenopausal women ($P = .480$ for interaction). When adjusted for age and other known breast cancer risk factors (age at menarche and parity, family history, history of cancer at other sites, body mass

index, alcohol use, and hormone use), the OR of carrying a diagnosis of breast cancer in estrogen receptor-positive women was 2.63 (95% CI = 1.47–4.70). For premenopausal women, the OR was 2.04 (95% CI = 0.97–4.3) and for postmenopausal women there was a stronger association between estrogen receptor positivity of breast epithelium and breast cancer, with an OR of 3.8 (95% CI = 1.5–9.8).

Effect of Increasing Estrogen Receptor Content

We next examined the possibility of an increasing effect on breast cancer risk with increasing estrogen receptor positivity. Crude and adjusted ORs for three different categories of estrogen receptor positivity are shown in Table 1. The test for trend in estrogen receptor positivity for all women was statistically significant ($P < .001$), although the crude ORs for women with estrogen receptor labeling index 1.00–4.99 and estrogen receptor labeling index greater than or equal to 5.00 were similar (3.1 and 3.2, respectively). With the inclusion of additional covariates, the OR estimates for increasing levels of estrogen receptor positivity remained elevated but nonsignificant in premenopausal women. However, a statistically significant increase in the estimated relative risk of developing breast cancer in postmenopausal women was

found with the inclusion of covariates. The adjustment for other study covariates (history of lactation, recent birth, history of radiation, smoking, marital status, education, and ethnicity) did not appreciably alter the final ORs.

Effect of Menstrual Cycle Variation

Since estrogen receptor expression in breast epithelium has been described by several authors to vary with the menstrual cycle in premenopausal women (16,17), we examined the effect of timing of sampling relative to the menstrual cycle in premenopausal women. This analysis is shown in Fig. 1, where the median estrogen receptor positivity of case subjects is compared with the median value for control subjects. Each phase contained approximately one-third case subjects and two-thirds control subjects, although the absolute numbers of women in each phase varied from 14 to 76 (Fig. 1). The difference in median estrogen receptor labeling index differed significantly in the 25–28-day interval ($P = .032$); the difference did not reach statistical significance in the other intervals shown, probably because of small numbers. The data suggest that case subjects and control subjects demonstrate opposite trends in the estrogen receptor positivity as the menstrual cycle progresses. In women without breast cancer, an expected decreasing trend in es-

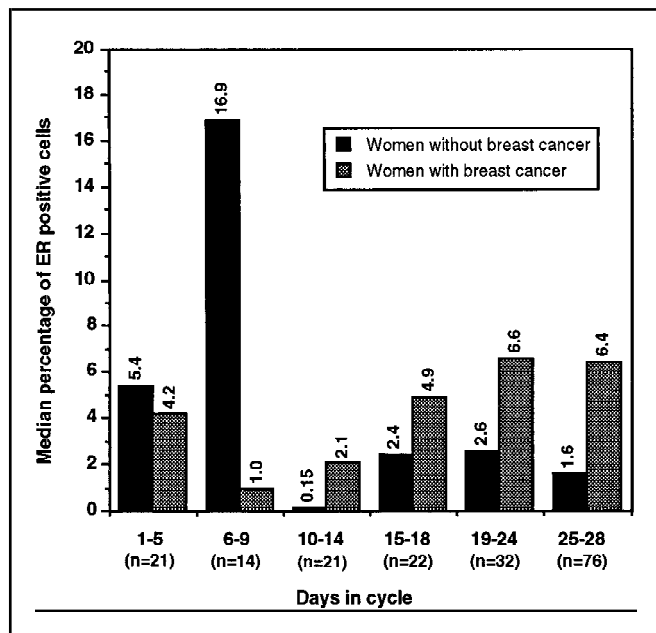
Table 1. Crude and adjusted ORs for breast cancer risk in relation to increasing levels of ER positivity*,†

	All women				Premenopausal				Postmenopausal			
	Case subjects	Control subjects	OR	95% CI	Case subjects	Control subjects	OR	95% CI	Case subjects	Control subjects	OR	95% CI
Crude ORs												
ER LI												
0–0.99	25	70	1.00	—	13	54	1.00	—	12	16	1.00	—
1.00–4.99	47	43	3.1	1.6–6.0	27	36	3.1	1.3–7.4	20	7	3.8	1.1–14.1
≥5.00	102	89	3.2	1.8–5.7	30	59	2.1	0.9–4.8	72	30	3.2	1.3–8.3
		P trend <.001				P trend = .085				P trend = .018		
Adjusted ORs												
ER LI												
0–0.99	25	70	1.00	—	13	54	1.00	—	12	16	1.0	—
1.00–4.99	47	43	3.03	1.6–5.9	27	36	2.2	1.0–5.2	20	7	4.4	1.3–14.5
≥5.00	102	89	2.19	1.2–4.0	30	59	1.57	0.7–3.5	72	30	3.1	1.3–7.6
		P trend = .029				P trend = .384				P trend = .032		
ER LI												
0–0.99	17	59	1.0	—	9	44	1.0	—	8	15	1.0	—
1.00–4.99	34	35	3.7	1.6–9.2	21	29	2.7	0.9–8.4	13	6	4.4	0.8–25.8
≥5.00	78	60	3.0	1.3–6.9	24	43	1.8	0.6–5.2	54	17	7.3	1.8–29.5
		P trend = .015				P trend = .440				P trend = .005		

*ER = estrogen receptor; LI = labeling index; CI = confidence interval; OR = odds ratio.

†Table 1 shows the crude and adjusted ORs for increasing categories of estrogen receptor labeling index. The analyses are presented as crude ORs for the whole study population (all women) and for pre- and post-menopausal women separately. The first panel shows the crude ORs, the middle panel the ORs adjusted for age only, and the last panel the ORs adjusted for age, history of cancer other than of the breast, age at menarche, age at parity, body mass index, alcohol use, hormone use, and family history of breast cancer in a mother or sister.

Fig. 1. Median percentage of estrogen receptor (ER)-positive cells in each phase of a standardized 28-day menstrual cycle. Number of case subjects and control subjects in each phase is as follows: days 1–5, six case subjects and 15 control subjects; days 6–9, three case subjects and 11 control subjects; days 10–14, five case subjects and 16 control subjects; days 15–18, eight case subjects and 14 control subjects; days 19–25, 10 case subjects and 22 control subjects; and days 25–28, 24 case subjects and 52 control subjects.



trogen receptor expression in the latter part of the cycle was observed, as reported by others (16–19). In the case of women with breast cancer, estrogen receptor expression tended to increase with the progression of the menstrual cycle. ORs for breast cancer in relation to estrogen receptor positivity across the six phases of the menstrual cycle were unstable, but generally revealed an increasing trend from OR = 1.25 in days 1–5 to OR = 4.38 in days 25–28 (chi-squared trend = 5.90; $P = .015$).

The ORs for progesterone receptor positivity were not significantly different from unity; for all women, the OR was 0.996 (95% CI = 0.98–1.01). Progesterone receptor-positive premenopausal women exhibited an OR of 1.0 (95% CI = 0.99–1.02), and postmenopausal women exhibited an OR of 0.999 (95% CI = 0.98–1.02).

Interaction With Other Risk Factors

There were no significant first-order interactions between estrogen receptor positivity and other covariates, with case-control status as the outcome. In particular, there were no interactions with early menarche, late parity, or late menopause. There were 39 case subjects and 97 control subjects with a history of any oral contraceptive use in the past, and estrogen receptor positivity was equally distributed between them. Postmenopausal hormone use was relatively infrequent: 23 case subjects and 30 control subjects described

using HRT at any time, and only 14 case subjects and 20 control subjects were current users. Estrogen receptor positivity was significantly more frequent in nonusers if they were case subjects (88.6% versus 55.2%); but among the users of HRT, proportions of women showing estrogen receptor-positive epithelium was roughly equal, regardless of case-control status (85.7% versus 85.0%), as shown in Table 2. However, there was no significant interaction between current HRT use and estrogen receptor positivity in terms of breast cancer risk ($P = .106$).

Nulliparous interval was modeled as a continuous variable and was found to be a significant predictor of case status, with risk increasing by 6% for each additional year (OR = 1.06; 95% CI = 1.03–1.11). There was no significant interaction between nulliparous interval and estrogen receptor status of the benign epithelium. The fact that variations in nulliparous interval could result from oral contraceptive use in some women was not a point of analysis here.

Table 3 shows the characteristics of

women with estrogen receptor-positive and -negative tissue. The mean age of estrogen receptor-positive women was higher than those who were estrogen receptor negative. On the whole, there was a trend toward postmenopausal women being estrogen receptor positive more frequently than premenopausal women ($P < .07$), with a significant positive correlation between age and estrogen receptor positivity in the entire population ($r = .2$; $P < .001$). The effect of age on estrogen receptor positivity was no longer significant when women were stratified by menopausal status. Premenopausal women who consumed alcohol regularly were more frequently estrogen receptor positive (8.6%) than women who denied alcohol consumption (4.5%). Among postmenopausal women, estrogen receptor positivity was more likely if they were nulliparous, had had a prior cancer other than that of the breast (uterus, ovaries, colon, and lymphoma), had a mother or sister with breast cancer, were using HRT, or had a greater time interval from menarche to either first full-term pregnancy or menopause. Body mass index was not significantly different, both between case subjects and control subjects and between estrogen receptor-positive and -negative women.

Discussion

We have compared estrogen receptor alpha and progesterone receptor expression in normal, nonhyperplastic breast epithelium from breast cancer case subjects with that in control subjects with only benign disease. This model has the advantage of comparing preneoplastic epithelium from a high-risk group with a defined probability of developing new breast cancers (0.75% per year) (20) to similar epithelium from women whose future risk of breast cancer is close to that of the general population. We find that the odds of a woman with estrogen receptor-

Table 2. Prevalence of estrogen receptor positivity by hormone-replacement therapy (HRT) use and case subject status (postmenopausal women)

	Nonusers of HRT		Current HRT users	
	Control subjects	Case subjects	Control subjects	Case subjects
ER negative (%)	13 (44.8)	10 (11.4)	3 (15.0)	2 (14.3)
ER positive (%)	16 (55.2)	78 (88.6)	17 (85.0)	12 (85.7)
Total	29	88	20	14
	Pearson χ^2 (df, 1) = 15.47; $P = .0001$		Pearson χ^2 (df, 1) = 0.003; $P = .95$	

Table 3. Characteristics of estrogen receptor (ER)-positive and -negative women by menopausal status*

	Premenopausal women (n = 219)	Postmenopausal women (n = 157)
Mean age, y (SD)		
ER-positive women	39.0 (8.5)	60.8 (12.0)
ER-negative women	34.7 (7.5)	59.2 (10.6)
Mean age, y, at first full-term pregnancy (SD)		
ER-positive women	24.0 (5.3)	23.3 (4.7)
ER-negative women	24.6 (5.0)	23.4 (4.6)
Mean age, y, at menarche (SD)		
ER-positive women	12.4 (1.4)	12.6 (1.4)
ER-negative women	12.4 (1.8)	13.0 (1.8)
Mean years from menarche to either first full-term pregnancy or menopause (SD)		
ER-positive women	14.4 (8.0)	14.2 (10.0)
ER-negative women	14.2 (6.4)	12.6 (9.2)
Percentage nulliparous†		
ER-positive women	32.9	22.9
ER-negative women	33.3	15.4
Percentage with prior cancer at any site		
ER-positive women	10.0	18.0
ER-negative women	12.7	11.1
Percentage with a mother and/or sister diagnosed with breast cancer		
ER-positive women	20.0	21.9
ER-negative women	19.4	14.3
Mean body mass index (SD)		
ER-positive women	25.1 (5.4)	27.7 (6.2)
ER-negative women	25.3 (5.1)	26.4 (5.6)
Percentage using either hormone-replacement therapy or birth control pills		
ER-positive women	13.8	23.6
ER-negative women	13.8	17.9
Percentage with alcohol intake >3 times per week		
ER-positive women	8.6	7.8
ER-negative women	4.5	7.1

*SD = standard deviation; CI = confidence interval.

†Median age of nulliparous women was 31 years (95% CI = 28.2–34.8).

positive breast epithelium having a cancer of the breast are significantly elevated, with the crude OR being 3.16 (95% CI = 1.89–5.28) and the adjusted OR after controlling for known breast cancer risk factors (including age) being 2.63 (95% CI = 1.47–4.70). The effect of estrogen receptor positivity is stronger for postmenopausal than for premenopausal women, but it should be noted that estrogen receptor expression in premenopausal women varies with the menstrual cycle. Since our samples were not collected in a specific phase of the menstrual cycle, the random variation of surgical timing within the menstrual cycle may be diluting the effect of estrogen receptor positivity thus biasing our results toward the null in the premenopausal subset.

Progesterone receptor positivity was equally prevalent in the breast epithelium of case subjects and control subjects, with

no significant differences in the proportion of positive cells, with crude ORs in both premenopausal and postmenopausal women being very close to one. This is in agreement with our previous finding that progesterone receptor positivity is uniformly prevalent in most women and is a constant feature of normal breast epithelium (13) [reviewed in (21)].

The threshold for estrogen receptor and progesterone receptor positivity was prospectively chosen as 1%, since this level has been reported to correspond to the commonly used threshold of 10 fm/mg cytosol protein in breast cancer samples (14). Other thresholds for estrogen receptor positivity between greater than zero and 2% did not alter the ORs appreciably; a threshold of 5% resulted in lower ORs (data not shown). However, analysis using three categories of estrogen receptor expression (labeling index <1, 1–

4.99, and ≥ 5) shows that increasing proportions of estrogen receptor-positive cells result in higher ORs for postmenopausal women (P trend .005) but not for premenopausal women (Table 1). This difference in trends may be related to the variability of estrogen levels in premenopausal women, and needs to be examined further.

Estrogen receptor positivity of breast epithelium does not appear to be modulated by the endocrine risk factors, such as early menarche, late first-term pregnancy and late menopause, and does not explain the high risk associated with them. We found no significant interactions between estrogen receptor positivity and these risk factors in our study population, which contained 76 women with early menarche, 31 with late menopause, 32 with late first-term pregnancy, and 74 with nulliparity. Since the breast epithelium is particularly susceptible to transforming events between menarche and the terminal differentiation associated with term pregnancy, we collapsed the endocrine-related risk categories into one, defined above as the nulliparous interval (*see* “Methods” section). The odds of having breast cancer increased by 6% for each additional year that the breast epithelium was exposed to ovarian cycles without a term pregnancy, but the duration of the nulliparous interval did not influence estrogen receptor positivity in either case subjects or control subjects, and we found no interaction between nulliparous interval, estrogen receptor positivity, and the occurrence of breast cancer. These results were unaffected by censoring premenopausal nulliparous women who still have not completed their reproductive life span.

The use of HRT by postmenopausal women resulted in a dramatic increase in the proportion of estrogen receptor-positive control subjects, so that there was no difference between estrogen receptor-positivity rates between case subjects and control subjects in this subset (Table 2). This finding needs to be pursued further in a larger study, with pre- and post-HRT assessments of estrogen receptor expression. If confirmed, it would suggest that HRT promotes estrogen receptor positivity in postmenopausal women and may contribute to breast cancer risk through this mechanism.

Estrogen receptor expression in benign

breast epithelium has been described to decline late in the menstrual cycle. Our data are consistent with this finding in the control subjects, but in case subjects with breast cancer the reverse trend was seen, with median estrogen receptor labeling index increasing late in the cycle. Additionally, we observed a statistically significant trend for increasing odds of breast cancer in women whose breast epithelium was estrogen receptor positive late in the cycle ($P < .015$). These data suggest that dysregulated estrogen receptor expression, as reflected by estrogen receptor positivity in the luteal phase, carries a particularly strong association with the presence of breast cancer, and lead us to speculate that loss of the normal regulatory mechanisms that control expression of estrogen receptor in normal breast epithelium may confer an increased risk for the development of breast cancer. These findings also imply that future studies examining the relationship of estrogen receptor expression in benign breast epithelium and breast cancer risk must control for menstrual cycle dates in premenopausal women, and that estrogen receptor expression in the luteal phase deserves special attention.

In conclusion, further investigation of estrogen receptor expression in breast epithelium may identify points in the estrogen response pathway that may be interrupted to avoid the cancer-promoting effects of estrogen on breast epithelium. In that context, strategies that decrease estrogen receptor expression may prove protective against the carcinogenic effects of estrogen and other compounds collectively called xenoestrogens, which have affinity for estrogen receptors.

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Notes

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Causes of Cervical Cancer in the Philippines: a Case–Control Study

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Background: Among the numerous human papillomavirus (HPV) types, only types 16 and 18 have been formally classified as human carcinogens. To evaluate the associations of 33 HPV types and other risk factors with squamous cell carcinoma and adenocarcinoma of the cervix, we performed a hospital-based, case–control study in the Philippines. **Methods:** The study included 356 case subjects who had histologically confirmed cervical cancer (323 incident cases of squamous cell carcinoma and 33 incident cases of adenocarcinoma/adenosquamous carcinoma) and 381 control subjects. Information on risk factors was obtained by personal interview. HPV DNA was detected in exfoliated cervical cells and biopsy specimens by use of a polymerase chain reaction assay. **Results:** HPV DNA was detected in 93.8% of case subjects with squamous cell carcinoma and in 90.9% of case subjects with adenocarcinoma/adenosquamous carcinoma compared with 9.2% of control subjects, giving age-adjusted odds ratios of 156 (95% confidence interval [CI] = 87–280) for squamous cell carcinoma and 111 (95% CI = 31–392) for adenocarcinoma/adenosquamous carcinoma. Fifteen different HPV types were detected in squamous cell carcinoma, and six different HPV types were detected in adenocarcinoma/adenosquamous carcinoma. Among HPV types other than types 16 and 18, the associations of HPV with risk of squamous cell carcinoma were strongest for HPV45. In addition to HPV, high parity, low socioeconomic status,

and smoking were also associated with both types of cervical cancer. **Conclusions:** As has been shown for squamous cell carcinoma, HPV is the central cause of adenocarcinoma/adenosquamous carcinoma of the uterine cervix. The observed associations of less prevalent HPV types with cervical cancer have important implications for cervical cancer prevention strategies. [J Natl Cancer Inst 1998;90:43–9]

Certain types of human papillomavirus (HPV) are currently recognized as the central etiologic factor of invasive cervical cancer (1,2) and of its precursor lesions (3–5). In 1995, the International Agency for Research on Cancer (IARC) (6) evaluated all relevant data on the carcinogenicity of HPV and concluded that there was sufficient evidence to categorize HPV types 16 and 18 as human carcinogens but that the existing evidence was limited or inadequate for the other HPV types. At that time, odds ratios (ORs) for HPV types 31, 33, and 35 and invasive cervical carcinoma had been reported for only the three types as a group from case–control studies in Spain and Colombia (1,3) and in Brazil (2). Since the IARC evaluation, we have completed case–control studies in other populations in which standard protocols and questionnaires were employed. A polymerase chain reaction (PCR)-based assay capable of detecting 33 HPV types was used (7,8), and ORs for the most common HPV types were estimated for both squamous cell carcinomas and adenocarcinomas. The role of other risk factors after controlling for the strong effect of HPV has been examined in a few studies (1–3).

We report here the main results of a hospital-based, case–control study conducted in Manila, the Philippines, which has a population with intermediate rates for cervical cancer (age-adjusted incidence rate 25 per 100 000) (9).

Patients and Methods

Study Population

From April 1991 through April 1993, 387 consecutive women with a diagnosis of invasive cervical cancer and 392 control women were identified from the outpatient clinics and wards of the Philippine General Hospital in Manila. Inclusion criteria for case subjects were that they had a histologically confirmed cervical cancer (incident case of squa-

mous cell carcinoma or adenocarcinoma/adenosquamous carcinoma) and had not received any treatment. The histologic slides in which the diagnosis was made were reviewed by an expert pathologist (M. Santamaria). Control women were selected to match the expected age distribution of the case subjects and had to fulfill the following inclusion criteria: not to have had conization, hysterectomy, or diseases associated with known risk factors for cervical neoplasia (other anogenital tumors and tumors of the breast, oral cavity, esophagus, lung, bladder, and liver; cardiovascular or cerebrovascular diseases; chronic bronchitis; or emphysema). Evidence of cytologic abnormality detected on examination after recruitment was not a criterion for exclusion. Case and control subjects who were in poor physical or mental condition were excluded.

All eligible case subjects and 387 (98.7%) of 392 eligible control subjects agreed to participate and provided biologic specimens.

The cytologic diagnoses of the control subjects at entry into the study were as follows: normal (n = 277), inflammatory (n = 84), low-grade squamous intraepithelial lesion (n = 6), high-grade squamous intraepithelial lesion (n = 3), and inadequate for diagnosis (n = 12).

The main diagnostic categories of the control subjects included in the study were urinary infections (n = 75), benign disorders of the genital tract (n = 53), menstrual disorders (n = 43), diseases of the circulatory system (n = 31), mild mental disorders (n = 28), endocrine or metabolic disease (n = 18), diseases of the respiratory system (n = 16), diseases of the nervous systems (n = 14), diseases of the musculoskeletal system (n = 14), diseases of the digestive system (n = 9), diseases of the skin (n = 9), infectious or parasitic disease (n = 8), and other diseases (n = 12). In addition, 57 healthy women who were visiting the hospital for reasons other than illness (usually accompanying outpatients) were included as control subjects.

After histologic review, the cancers in the case subjects were classified as follows: squamous cell carcinoma (n = 344), adenocarcinoma (n = 23),

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adenosquamous carcinoma (n = 11), and clear cell carcinoma (n = 3); histologic slides were not available for six case subjects. Two main groups of carcinoma were considered for the analysis by histologic type: squamous cell carcinoma and adenocarcinoma/adenosquamous carcinoma. The squamous cell carcinomas were further categorized into the following subtypes: keratinizing large cells (2.4%), nonkeratinizing large cells (94.2%), nonkeratinizing small cells (0.5%), and others (2.9%).

Information on clinical stage was available for 371 (96%) of the case subjects. The stages (10) were distributed as follows: stage I, 62 case subjects (17%); stage II, 136 case subjects (37%); stage III, 170 case subjects (46%); and stage IV, three case subjects (0.01%).

Data and Specimen Collection

Study subjects were interviewed at the hospital by use of a standardized questionnaire to elicit information on sexual behavior, reproductive history, contraceptive practice, smoking habits, genital hygiene, history of sexually transmitted diseases, screening history, and various measures of socioeconomic status (e.g., education, occupation, income per capita, type of housing, and household facilities). Three specially trained female technicians administered the interview to all case and control subjects. An effort was made to keep them blinded to the case or control status of the study subjects. The average time taken to complete the interview was 23 minutes. After the interview, all women had a pelvic examination performed by a gynecologist, who also took two cervical scrapes with an Ayre spatula and an endocervical brush. One Pap smear was prepared, and the remaining cells were eluted in phosphate-buffered saline (PBS), pelleted in PBS (2000 rpm for 10 minutes at room temperature), and kept at -70°C for later use for HPV DNA detection. A tumor biopsy specimen was also taken in most cases and kept frozen for virologic studies. A 10-mL blood sample was also collected from all women for the detection of antibodies to certain sexually transmitted agents.

The study protocol was cleared by the ethical committee of the International Agency for Research on Cancer and by local ethical committees. Written informed consent was obtained from the study subjects.

HPV DNA Detection and Typing

HPV DNA detection in cervical scrapes was performed by PCR (7,8). To analyze the quality of target DNA for PCR purposes, we prescreened specimens by PCR with the use of β -globin gene-specific oligonucleotide primers (11). Specimens that showed successful amplification of β -globin sequences were subjected to HPV DNA genotyping.

The genotyping was carried out as follows: Briefly, a first screening to determine the overall presence of HPV was performed by use of a general primer GP5+/6+-mediated PCR, which permits the detection of a broad spectrum of sequenced and still-unsequenced genital HPV types at the subpicogram level (7). HPV positivity was assessed by low-stringency Southern blot analysis of PCR products with a cocktail probe of HPV-specific DNA fragments (12). Subsequently, GP5+/6+ PCR was repeated in triplicate on positive samples to generate

sufficient products for further typing. PCR products from independent reactions were pooled to generate 11 identical Southern blots for parallel and successive hybridization rounds for typing with the use of internal type-specific oligoprobes (8). The strategy of melting and rehybridization of the filters was essentially the same as that described earlier (13). The first hybridization round was performed for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, and 56. The second round included HPV types 6, 11, 26, 34, 40, 42, 43, 44, 58, 59, and 68. The third round was performed for HPV types 57, 61, 66, 70 (equivalent to CP141-7), 72 (equivalent to CP4137), 73 (equivalent to MM9), IS39 and MM4 (related to HPV51), MM7, CP6108, and CP8304. The last three sequences are phylogenetically related to the low-risk HPV61 and HPV62 (12,13). Type-specific oligonucleotides (30-mers) were selected on the basis of sequence information from the HPV sequence database (14,15) after alignment analysis with the CLUSTAL program (PC/Gene Release 6.7; Intelligenetics, Inc., Mountain View, CA) (16).

In a second step, for those specimens found to be β -globin PCR negative as well as HPV PCR negative, DNA was extracted from the cell pellets and retested for HPV DNA as described above. In addition, for those cases for which not enough cell pellet was available as well as for those cases testing negative for HPV in the exfoliated cells, the HPV DNA analysis was done on the corresponding biopsy specimens of these carcinomas. For HPV detection in the biopsy specimen, the sandwich method was used as described earlier (17). Briefly, snap-frozen tissues were cut in a way that the outer sections of a series of sections were used for histologic analysis, and the inner sections were used for PCR testing.

The special precautions we took to minimize false-positive results in the PCR have been described in detail elsewhere (12).

Only those specimens found to be β -globin PCR positive or negative and HPV DNA positive were included in the analysis. Thus, 22 (5%) case subjects and six (1.5%) control subjects classified as negative for β -globin and HPV DNA were excluded, which left 365 case subjects and 381 control subjects. In addition, six case subjects for whom the histologic diagnosis was not confirmed and three who had a diagnosis of clear cell carcinoma were also excluded, which left a final total of 356 case subjects.

For case subjects, the combined prevalence of HPV DNA (detected in exfoliated cells and in biopsy specimens) is given. For control subjects, the HPV DNA prevalence was detected only in exfoliated cells.

Statistical Analysis

To estimate the risk of cervical cancer associated with various HPV types and the other risk factors, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) as approximations of relative risks by using unconditional logistic regression (18). The ORs for HPV were adjusted only for age (categorized in four age groups: <40 years, 40-49 years, 50-59 years, and \geq 60 years). ORs for other risk factors are given, adjusted for age and for age, HPV, and all variables included in the final model. In an attempt to identify factors that influence the progression from HPV carrier state to invasive carcinoma, we used a model that was restricted to HPV-positive

case and control subjects and that included all variables being evaluated.

We examined various measures of socioeconomic status: place of residence, educational level, occupation, housing characteristics, and number of household amenities (i.e., television, refrigerator, and toilet and running water inside the house). Educational level and household amenities showed the strongest associations with risk, but only the latter was included in the final multivariate analysis.

Statistical significance was tested by use of the likelihood ratio test for the difference for dichotomous variables and for two-sided linear trend for variables with logically ordered categories.

From the variables that were highly correlated (with the use of Pearson's correlation coefficients) and that may have been measuring the same effect, one was selected for inclusion in the final model; i.e., age at first intercourse that was highly correlated with age at first marriage and age at first pregnancy (correlation coefficients = .97 and .84, respectively) was included in the final model.

HPV attributable fractions (AFs) have been calculated according to standard methods, $AF = p(r - 1) / [1 + p(r - 1)]$, where p = HPV DNA prevalence in the population approximated by the prevalence in the control group and r = relative risk approximated by the OR.

Some deviations from the protocol could have introduced biases in the selection of control subjects. The design was hospital based, but 57 control subjects were healthy women who were visiting the hospital for reasons other than illness, usually accompanying a sick relative. In addition, 53 women with benign disorders of the genital tract, who were ineligible according to our protocol, were included. However, the exposures to the risk factors under study of these two groups of women were compared with those of the other hospital control subjects, and no significant differences were found. Moreover, the prevalence of cytologic abnormalities and HPV positivity in these two groups was very similar to that of the whole group of control subjects. Finally, excluding these two groups of women from the analyses had little effect on the adjusted risk estimates for HPV and other risk factors; therefore, they were included in the final analyses.

Results

For patients with squamous cell carcinoma, the mean age was 47.2 years; for those with adenocarcinoma/adenosquamous carcinoma, it was 48.4 years; for control subjects, it was 46.8 years.

Table 1 summarizes the HPV DNA prevalence in case and control subjects.

Among control subjects, the HPV DNA prevalence in exfoliated cells was 9.2%. It increased with the number of sexual partners and was lower among women starting sexual intercourse under 20 years of age, but the tests for trend were not statistically significant. However, the HPV DNA prevalence did not show a clear trend with age or education. Among case subjects, the HPV DNA

Table 1. Human papillomavirus (HPV) types in women with cervical cancer and in control women in the Philippines*

HPV type	Squamous cell carcinoma		Adenocarcinoma/ adenosquamous carcinoma		Control subjects	
	No.	%	No.	%	No.	%†
Negative	20	6.2	3	9.1	346	90.8
Positive for any HPV type.....	303	93.8	30	90.9	35	9.2
Total.....	323	100.0	33	100.0	381	100.0
HPV6					3	8.6
HPV11					1	2.9
HPV16	110	36.3	4	13.3	5	14.3
HPV18	61	20.1	15	50.0	4	11.4
HPV31	2	0.7			2	5.7
HPV39	1	0.3				
HPV45	37	12.2	3	10.0	4	11.4
HPV51	6	2.0				
HPV52	9	3.0			2	5.7
HPV54					1	2.9
HPV56	2	0.7				
HPV58	9	3.0				
HPV59	5	1.7	1	3.3		
HPV68	2	0.7				
HPV8304					3	8.6
HPV X	34	11.2	2	6.7	5	14.3
HPV16 + HPV18	12	4.0	2	6.7		
HPV16 + HPV18 + HPV51	1	0.3				
HPV16 + HPV18 + HPV66	1	0.3				
HPV16 + HPV45	2	0.7	2	6.7		
HPV16 + HPV51	1	0.3				
HPV16 + HPV66	2	0.7				
HPV16 + HPV73	1	0.3				
HPV18 + HPV45	1	0.3			1	2.9
HPV18 + HPV51	1	0.3				
HPV31 + HPV59			1	3.3		
HPV45 + HPV52	1	0.3				
HPV45 + HPV58					1	2.9
HPV51 + HPV66	1	0.3				
HPV44 + HPV6108					1	2.9
HPV58 + IS39 + MM4					1	2.9
HPV IS39 + MM4	1	0.3				
HPV40 + HPV56 + HPV73 + HPV8304 + MM4					1	2.9
Total multiple types	25	8.3	5	16.7	5	14.3

*Percentage of HPV types are calculated relative to the total HPV positive and indicates percentage of positive cases in which a specific type alone or in combination with other types was detected.

†Beginning with the fourth value in this column (i.e., 8.6), all percentages reflect the percent of HPV-positive control subjects.

prevalence among case subjects did not vary with any of the variables considered (data not shown).

Fifteen different HPV types were detected among the patients with squamous cell carcinoma, five among the patients with adenocarcinoma/adenosquamous carcinoma, and 17 among the control subjects. HPV types 6, 11, 26, 33, 34, 35, 40, 42, 43, 44, 54, 57, 61, MM7, and CP4173 were not identified in any of the tumor specimens from patients with squamous cell carcinoma (Table 1).

The most common HPV type in women with squamous cell carcinoma was HPV type 16 followed by HPV types 18, 45, 52, and 51; in women with adenocarcinoma/adenosquamous carcinoma, HPV type 18 was the most common type followed by types 16 and 45.

HPV type 45 was the most common type in control women followed by HPV types 16 and 18.

Two or more different HPV types were detected in 25 (7.7%) of the women with squamous cell carcinoma, in five (15.2%) of the women with adenocarcinoma/adenosquamous carcinoma, and in five (1.3%) of the control subjects; these are counted twice or more in Table 2. Thirty-two (9.9%) specimens from patients with squamous cell carcinoma, two (6.1%) from patients with adenocarcinoma/adenosquamous carcinoma, and five (1.3%) from control women had HPVs that could not be typed with the 33 type-specific probes that we used and were classified as HPV X. The age-adjusted ORs for the most common HPV types (those detected in at least four case sub-

jects) are given in Table 2. For any HPV, the age-adjusted OR for squamous cell carcinoma was 156 and the AF was 93.5%; for adenocarcinoma/adenosquamous carcinoma, the OR was 111 and the AF was 91%.

For both squamous cell carcinoma and adenocarcinoma/adenosquamous carcinoma, very high ORs were observed with the various HPV types. In squamous cell carcinoma, they ranged from 81 for HPV58 to 506 for HPV16; in adenocarcinoma/adenosquamous carcinoma, they ranged from 259 for HPV45 to 948 for HPV18.

The association of cervical cancer risk with HPV66 cannot be properly assessed because it was detected only as coinfection with other high-risk HPV types.

Table 3 summarizes the association of

Table 2. Number and percentage of human papillomavirus (HPV)-positive case and control subjects and odds ratios (ORs) for association between squamous cell carcinoma and adenocarcinoma/adenosquamous carcinoma of the cervix and HPV types

HPV type	Case subjects						OR* (95% confidence interval)	
	Squamous cell carcinoma		Adenocarcinoma/ adenosquamous carcinoma		Control subjects		Squamous cell carcinoma	Adenocarcinoma/ adenosquamous carcinoma
	No.	Percentage of HPV positive	No.	Percentage of HPV positive	No.	Percentage of HPV positive		
Negative	20	6.2	3	9.1	346	90.8	1.0 (referent)	1.0 (referent)
Positive for any HPV type	303	93.8	30	90.9	35	9.2	156 (87-280)	111 (31-392)
Total	323	100.0	33	100.0	381	100.0		
HPV16	130	40.2	8	24.2	5	1.3	506 (178-1436)	549 (44-6912)
HPV18	77	23.8	17	51.5	5	1.3	276 (99-771)	948 (97-9240)
HPV45	41	12.7	5	15.2	6	1.6	124 (46-335)	259 (26-2618)
HPV51	10	3.1			0	0.0	∞ (44.7-∞)	
HPV52	10	3.1			2	0.5	93 (18-484)	
HPV58	9	2.8			2	0.5	81 (14-480)	
HPV59	5	1.5			0	0.0	∞ (19.3-∞)	
HPV66	4	1.2			0	0.0	∞ (14-∞)	
HPV X	34	10.5	2	6.1	5	1.3	111 (39-317)	50 (2-456)
Other types	11	3.4	2	6.1	13	3.4	16.0 (6.1-41.5)	15 (2-116)
Multiple types†	25	7.7	5	15.2	5	1.3	91 (31-271)	150 (23-973)

*Adjusted for age.

†Included in the calculation of prevalence and ORs for individual types.

Table 3. Association of squamous cell carcinoma of the cervix with risk factors other than human papillomavirus (HPV)

Risk factors	Case subjects	Control subjects	All women		HPV-positive women
			Odds ratio* (95% confidence interval)	Odds ratio† (95% confidence interval)	Odds ratio‡ (95% confidence interval)
No. of household amenities§					
4	75	170	1.0 (referent)	1.0 (referent)	1.0 (referent)
3	87	98	2.0 (1.4-3.0)	1.6 (0.7-3.7)	1.5 (0.5-4.8)
2	85	63	3.1 (2.0-4.7)	3.9 (1.4-10.4)	1.4 (0.4-4.8)
0-1	76	50	3.5 (2.2-5.5)	3.4 (1.2-9.8)	2.9 (0.5-15.1)
Age at first intercourse, y					
≥24	48	119	1.0 (referent)	1.0 (referent)	1.0 (referent)
21-23	61	93	1.6 (1.0-2.6)	2.1 (0.7-6.0)	2.3 (0.6-9.1)
18-20	102	112	2.3 (1.5-3.5)	1.9 (0.7-5.3)	1.9 (0.5-7.3)
<18	112	56	5.0 (3.1-7.9)	2.5 (0.9-7.3)	2.0 (0.5-7.7)
No. of live births					
0	5	25	1.0 (referent)	1.0 (referent)	1.0 (referent)
1-2	48	70	3.5 (1.3-9.9)	3.8 (0.6-24.0)	12.5 (1.2-127.0)
3-5	137	176	4.3 (1.6-11.8)	4.9 (0.8-30.0)	5.7 (0.7-45.0)
≥6	133	110	7.9 (2.9-22.0)	9.0 (1.2-68.0)	27.2 (2.2-237.0)
Lifetime No. of sexual partners					
1	248	341	1.0 (referent)	1.0 (referent)	1.0 (referent)
2	57	32	2.5 (1.5-3.9)	1.2 (0.4-3.3)	2.1 (0.5-9.1)
≥3	18	7	3.5 (1.4-8.6)	3.4 (0.4-29.0)	1.3 (0.1-18.0)
Use of hormonal contraceptives, y					
Never	258	277	1.0 (referent)	1.0 (referent)	1.0 (referent)
1-3	40	80	0.5 (0.3-0.8)	0.3 (0.1-0.7)	0.25 (0.08-0.8)
≥4	25	23	1.1 (0.6-2.1)	2.0 (0.5-7.6)	2.8 (0.2-30.0)
Unknown	0	1	—		
Smoking status					
Never	255	352	1.0 (referent)	1.0 (referent)	1.0 (referent)
Ever	68	29	3.3 (2.1-5.2)	11.2 (3.9-32.0)	6.6 (0.8-55.0)
Interval since last Pap smear, y					
Never	292	231	1.0 (referent)	1.0 (referent)	1.0 (referent)
≥5	13	34	0.3 (0.15-0.6)	0.2 (0.06-0.8)	0.5 (0.09-2.6)
<5	10	69	0.1 (0.05-0.2)	0.1 (0.04-0.05)	0.07 (0.02-0.3)

*Adjusted for age.

†Adjusted for age, HPV, and all variables in the table.

‡Adjusted for age and all variables in the table.

§Household amenities (television, refrigerator, and toilet and running water inside the house).

||A few were injectable hormonal contraceptives.

squamous cell carcinoma with risk factors other than HPV. After adjustment for age, HPV, and all factors shown in the table, the following factors were significantly related to risk: number of household amenities (P for trend = .003), smoking (P for trend = .001), and parity and age at first intercourse (borderline significance, P for trend = .07). With regard to smoking, a statistically significant increase in risk was observed with years of smoking and numbers of cigarettes smoked per day (data not shown). The effect of number of sexual partners decreased and became nonsignificant after adjustment for HPV (P for trend = .7). There was a strong protective effect with the interval since the last Pap smear (P for trend = .001). When a similar analysis was performed and was restricted to HPV-positive women only, similar associations were observed, but the tests for trend were statistically significant only for parity and interval since last Pap smear (P = .05 and .001, respectively).

Table 4 shows similar analyses for adenocarcinoma/adenosquamous carcinoma. After adjustment for age, HPV, and other factors included in the table, statistically significant associations were observed for number of household amenities (P for trend = .005), parity (P for trend = .03), and interval since last Pap smear (P for trend = .04). Among HPV-positive women, similar associations were observed, but only number of household amenities showed a statistically significant trend (P = .02).

Discussion

Of the 75 HPV types that have been cloned to date, at least 30 are known to infect the genital tract and 18 of them have been detected in frozen biopsy specimens from about 1000 women from 22 countries who have been diagnosed with invasive cervical cancer (19). Although experimental and some epidemiologic evidence suggests that these types

are carcinogenic, an estimation of the risk of developing cervical cancer linked to these HPV types can be derived only from case-control studies. In 1995, an IARC working group (6) evaluated the carcinogenicity of HPV types and concluded that only HPV16 and HPV18 could be classified as human carcinogens; other HPV genital types were classified as probable or possible carcinogens because the epidemiologic evidence available at that time was limited or inadequate.

By the application of the HPV general primer GP5+/6+-mediated PCR in combination with HPV type-specific oligoprobes, most, if not all, genital HPVs can be detected (7,8). The strategy used for typing in this study has been tested before (13), and it has been shown that three rounds of melting and rehybridization of the filters do not affect the sensitivity of the assay. Therefore, eventual underrepresentations of HPV types tested in this study are unlikely to be the result of the typing methodology. Using

Table 4. Association of adenocarcinoma/adenosquamous carcinoma of the cervix with risk factors other than human papillomavirus (HPV)

Risk factor	Case subjects	Control subjects	All women		HPV-positive women
			Odds ratio* (95% confidence interval)	Odds ratio† (95% confidence interval)	Odds ratio‡ (95% confidence interval)
No. of household amenities§					
3-4	10	268	1.0 (referent)	1.0 (referent)	1.0 (referent)
2	8	63	3.5 (1.3-9.3)	8.4 (1.1-63.2)	6.4 (0.6-63.0)
0-1	15	50	9.2 (3.8-22.0)	19.2 (2.3-156.0)	23.0 (1.4-392.0)
Age at first intercourse, y					
≥24	6	119	1.0 (referent)	1.0 (referent)	1.0 (referent)
21-23	7	93	1.4 (0.5-4.5)	0.9 (0.1-8.9)	1.9 (0.1-37.0)
18-20	11	112	2.0 (0.7-5.6)	0.2 (0.02-1.9)	0.6 (0.04-7.4)
<18	9	56	3.1 (1.0-9.1)	0.5 (0.06-5.1)	0.7 (0.03-15.8)
No. of live births					
0	1	25	1.0 (referent)	1.0 (referent)	1.0 (referent)
1-2	4	70	1.5 (0.16-13.8)	4.0 (0.07-210.0)	4.2 (0.05-339.0)
3-5	13	176	1.8 (0.2-14.7)	26.6 (0.6-1094.0)	19.9 (0.2-1855.0)
≥6	15	110	4.0 (0.5-33.0)	327.0 (3.9-27 176.0)	115.1 (1.2-1 106 540.0)
Lifetime No. of sexual partners					
1	22	341	1.0 (referent)	1.0 (referent)	1.0 (referent)
≥2	11	39	4.3 (1.9-9.6)	4.0 (0.5-30.0)	1.5 (0.1-17.0)
Use of hormonal contraceptives, y					
Never	26	277	1.0 (referent)	1.0 (referent)	1.0 (referent)
1-3	3	80	0.4 (0.1-1.3)	0.04 (0.003-0.5)	0.04 (0.001-1.1)
≥4	4	23	1.9 (0.6-5.9)	4.3 (0.3-57.0)	4.7 (0.3-81.0)
Unknown	0	1	—	—	—
Smoking status					
Never	31	352	1.0 (referent)	1.0 (referent)	1.0 (referent)
Ever	2	29	0.9 (0.2-4.1)	4.7 (0.2-129.0)	13.6 (0.07-2599.0)
Interval since last Pap smear, y					
Never	27	299	1.0 (referent)	1.0 (referent)	1.0 (referent)
≥1	2	103	0.16 (0.04-0.7)	0.1 (0.02-0.96)	0.1 (0.01-1.1)

*Adjusted for age.

†Adjusted for age, HPV, and all variables in the table.

‡Adjusted for age and all variables in the table.

§Household amenities (television, refrigerator, and toilet and running water inside the house).

||A few were injectable hormonal contraceptives.

this methodology, we report here the results of a case-control study conducted in the Philippines in which the risk for the seven HPV types more common in this population (HPV types 16, 18, 45, 51, 52, 58, and 59) was estimated. In addition, we were able to estimate the risk linked to some of these HPV types for the two main histologic types of cervical cancer: squamous cell carcinoma and adenocarcinoma/adenosquamous carcinoma. For both histologic types, a very strong association was observed for the presence of HPV DNA; the age-adjusted OR for squamous cell carcinoma was 156 and that for adenocarcinoma/adenosquamous carcinoma was 111. HPV16 was the most common type in squamous cell carcinoma (43% of the positives), followed by HPV18 (25%) and HPV45 (14%). In specimens of adenocarcinoma/adenosquamous carcinoma, HPV18 was the predominant type (57%), followed by HPV16 (27%) and HPV45 (17%). These distributions are similar to those reported in the IARC international survey of HPV types in invasive cervical cancer (19), with the exception of HPV types 33 and 35 that were not detected in our study population. However, these two types were also rare in the specimens from Southeast Asia included in our international survey; their prevalences were 2% and 1%, respectively.

Among control subjects, the three most common types of HPV were also the most commonly detected in cervical cancer, i.e., HPV types 16, 18, and 45, which has also been the case in our previous case-control studies in Colombia and Spain (1,3) and Brazil (2).

The age-adjusted ORs for squamous cell carcinoma were as follows: HPV type 16, OR = 506; HPV type 18, OR = 276; HPV type 45, OR = 124; and HPV types 51, 52, 58, and 59, ORS = >80. For adenocarcinoma/adenosquamous carcinoma, the ORs were as follows: HPV type 16, OR = 549; HPV type 18, OR = 948; and HPV type 45, OR = 259. These extremely high ORs are exceptional in cancer epidemiology.

It can be argued that these ORs have been artificially increased because those case subjects whose exfoliated cells were negative for HPV were tested again by use of the biopsy specimens, whereas the exfoliated cells of the control subjects

were tested only once. However, several observations justify the use of two types of biologic specimens in case subjects and one in control subjects. Our unpublished results from further testing of the specimens originally classified as negative in the IARC international survey of HPV types in invasive cervical cancer indicate that the true prevalence of HPV in these cancers is at least 99%. Concerning the HPV prevalence among control subjects, preliminary results from our ongoing study comparing the HPV prevalence in exfoliated cells with that detected in biopsy specimens taken from women without cervical neoplasia who were undergoing hysterectomy for various reasons indicate that the HPV prevalence is very similar in the two types of specimens. This result suggests that the use of only exfoliated cells in the control subjects does not underestimate the prevalence of HPV DNA.

The prevalence in the group of control subjects in the Philippines (9%) is lower than that reported in the control subjects in a study from Brazil (17%) in which the GP5/6 PCR-based assay was used (2). This result correlates well with the risk of cervical cancer in these two populations [age-adjusted incidence rate = 20 per 100 000 in Manila and 35 per 100 000 in São Paulo, Brazil (9)].

Although several case series in which PCR-based assays were used have reported a prevalence of HPV DNA ranging from 25% to 96% in adenocarcinoma (6), no formal case-control studies have been reported. To our knowledge, this is the first case-control study in which the risk linked to specific HPV types has been estimated for adenocarcinoma/adenosquamous carcinoma. Our results confirm that, as was found for squamous cell carcinoma, certain HPV types are the main cause of adenocarcinoma/adenosquamous carcinoma.

The main implications of our HPV findings are as follows: 1) In addition to HPV types 16 and 18, HPV types 45, 51, 52, 58, and 59 can now be considered to be carcinogenic for humans, if we follow the criteria used for the IARC monograph to evaluate the carcinogenicity. 2) HPV types 16, 18, and 45 are the main cause of adenocarcinoma/adenosquamous carcinoma in the Philippines. 3) The classification of HPV into high-risk, intermediate-risk, and low-risk types, as proposed a

few years ago by Lörintz et al. (20), based on the magnitude of the ORs should be revised.

Few independent risk factors were identified in addition to the strong effect of HPV. For squamous cell carcinoma, high parity, number of household amenities (as a surrogate of socioeconomic status), and interval since last Pap smear were significantly associated with risk. Similar associations have been observed in our studies in Spain and Colombia (1,3,21), Brazil (2), and Morocco (22) but not in Thailand (23). Although an association of risk of squamous cell carcinoma with early age at first intercourse was observed, it was not statistically significant. The statistically significant association of risk of squamous cell carcinoma with smoking is intriguing in this population in which the prevalence of smokers is very low (21% of the case subjects and 8% of the control subjects were ever smokers). No clear association of risk of squamous cell carcinoma with smoking was observed in our previous studies in Spain, Colombia, Brazil, Morocco, and Thailand.

Similar associations were observed for adenocarcinoma/adenosquamous carcinoma, but they were statistically significant only for number of household amenities, parity, and interval since last Pap smear.

The proper evaluation of risk factors other than HPV will require a pooled analysis of all case-control studies that we have carried out or are carrying out in various countries.

The validity or reliability of the assays for HPV DNA detection is critically important in studies on HPV and cervical neoplasia (24,25). The PCR-based assay that we have used in our study is considered today to be one of the most reliable (6). Design limitations should also be considered in the interpretation of our results. Our study was hospital based; as such, it may suffer from selection bias. The case subjects were consecutive incident case subjects attending the largest hospital in Manila, and all agreed to participate. The control subjects were attending the same hospital; therefore, it can be assumed that they came from the same population as the case subjects. However, there were some deviations from the protocol. The control group was intended to have a wide range of diagnoses that were

unrelated to the risk factors for cervical carcinoma, but a group of women with benign gynecologic conditions (n = 53) and a group of healthy women (n = 57) were included. However, the distribution of the various risk factors in these two groups was similar to that of the rest of the control subjects, and their exclusion from the analysis did not change the risk estimates for HPV and other risk factors. Besides, when we are dealing with ORs of the magnitude found in this study, bias or confounding is very unlikely to explain the very strong associations with the various HPV types.

In conclusion, in the Philippines as in other countries, cervical cancer is a late sequelae of infection with certain types of HPV. Likely cofactors are high parity, smoking, and unidentified factors linked to low socioeconomic status. Cervical cytology screening offers a strong protection.

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Notes

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Risk Factors for Cervical Cancer in Thailand: a Case-Control Study

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Background: Human papillomaviruses (HPV) types 16 and 18 are clearly involved in the etiology of cervical cancer, but the evidence for the carcinogenicity of other HPV types is limited. Cofactors involved in the progression from infection with HPV to high-grade precursors and cancer have not been clearly defined by the results of previous studies. **Methods:** We conducted a hospital-based, case-control study of invasive cervical cancer to investigate risk in relation to HPV infection and its epidemiologic cofactors in Hat-Yai, Thailand. A total of 338 patients with squamous cell carcinoma, 39 patients with adenocarcinoma/adenosquamous carcinoma, and 261 control subjects were included in the study and were interviewed to obtain information with regard to cervical cancer risk factors. HPV DNA presence in cervical exfoliated cells or frozen biopsy specimens was determined by a polymerase chain reaction assay. **Results:** HPV DNA was detected in 95% of patients with squamous cell carcinoma, 90% of those with adenocarcinoma/adenosquamous carcinoma, and 16% of control subjects. For patients with squamous cell carcinoma, the most common types of HPV found were type 16 (60% of the positives), type 18 (18%), type 58 (3%), type 52 (3%), and type 31 (2%). For patients with adenocarcinoma/adenosquamous carcinoma, the most common HPV types found were type 18 (60% of the positives), type 16 (37%), and type 45 (3%). The risk factors that remained associated with risk of both

histologic types after adjustment for HPV and their mutual confounding effects were limited education, increasing number of sexual partners, history of venereal diseases, and interval since last Pap smear (i.e., cytologic) test. Among patients with squamous cell carcinoma, some association with smoking was also observed. **Conclusion:** New preventive strategies for cervical cancer will require the consideration of multiple HPV types. [J Natl Cancer Inst 1998;90:50-7]

Several types of human papillomavirus (HPV), particularly types 16 and 18, are recognized as the main cause of cervical cancer and its precursor lesions (1). These two viral types were found in more than 60% of a large series of cases of invasive cervical cancers reported from 22 countries around the world (2). In that study, in all areas investigated, except Indonesia, HPV16 predominated in squamous cell carcinomas, whereas HPV18 often predominated in adenocarcinomas (2-4). Other viral types have been detected in cervical cancers, but no case-control studies have adequately investigated type-specific associations with risk to classify these other types as definite carcinogens (1). In addition, HPV infection appears to be a necessary but not a sufficient cause of cervical cancer, but the role of specific viral, host, or environmental factors for progression from infection to invasive disease has not been clarified.

When designing vaccine strategies or incorporating HPV testing in screening programs, it will be helpful to know the specific types of HPV operating as cervical carcinogens in different regions in order to know whether it is necessary to tailor interventions to the specific needs of particular areas. The definition of cofactors can help identify groups at particularly higher risk and can lead to new preventive strategies. To better define these issues, the International Agency for Research on Cancer (IARC) is conducting a series of case-control studies in several regions of the world, ensuring standardized epidemiologic methods and centralized polymerase chain reaction (PCR)-based HPV detection.

In this report, we present results from one of the studies conducted in the district of Hat-Yai, Province of Songkla in the

south of Thailand, an area with an estimated age-adjusted incidence rate of cervical cancer of 18.5 per 100 000 women (5).

Subjects and Methods

Study Population

Recruitment of study subjects was conducted from September 1990 through March 1993. Case subjects were women with invasive cervical cancer that was newly diagnosed and histologically or cytologically confirmed at the participating hospital in Hat-Yai. These subjects had not received previous treatment for cervical cancer, and all were in a sufficiently good physical and mental condition to provide reliable answers. Histologic and cytologic slides were reviewed by an expert pathologist (M. Santamaria). Control subjects were women without cervical cancer who were selected from the same hospital as the case subjects; these women were without histories of treatment with conization or hysterectomy and were broadly stratified by age (in 10-year groups) to the expected distribution of the case subjects. In addition, as was required for the case subjects, control subjects had to be in sufficiently good physical and mental condition to provide reliable answers. Other reasons for ineligibility of control subjects included diagnoses of anogenital tract cancers (vulva, vagina, or rectum), cancer of the breast, endometrium, ovary, or colon, benign genital tumors, and tobacco-related diseases (e.g., coronary heart disease, lung cancer, or chronic bronchitis).

The main diagnostic categories for the 354 control subjects originally recruited for the study were as follows: diseases of the digestive system (n = 89; 25%); endocrine, nutritional, or metabolic disorders (n = 83; 23%); diseases of the genitourinary system

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(n = 55; 16%); nongenital neoplasms (n = 33; 9%); and diseases of the circulatory system (n = 24; 7%). Cytologic specimens were available from 331 (94%) of the control subjects; their diagnoses, which were not used as a criterion for exclusion, were normal (n = 233; 70%), inflammatory or reactive (n = 91; 28%), and cervical neoplasia (n = 7; 2%), including one suspicious of adenocarcinoma.

Data and Specimen Collection

Each participant was administered a standardized questionnaire on sexual behavior, reproductive history, contraceptive practices, smoking habits, genital hygiene, histories of sexually transmitted diseases, cervical cytologic screening histories, and socioeconomic status. Face-to-face interviews were conducted by three different specially trained interviewers. An effort was made to keep the interviewers unaware of the case-control status of the study participants. Interviews lasted, on average, 30 minutes, but they were longer for case subjects (33.4 minutes) than for control subjects (25.7 minutes).

A pelvic examination was performed on case subjects and control subjects to obtain exfoliated cells for the preparation of a Pap smear and determination of the presence of HPV. The material was collected by sampling the ectocervix with a wooden spatula and the endocervix with a cytobrush; the procedure was performed in duplicate. The collection instruments were rinsed in phosphate-buffered saline (PBS), and the cell suspension was centrifuged for 10 minutes at 2000 rpm and room temperature. The cell pellets were stored at -70°C in the field until they were shipped to Lyon, France, or to Amsterdam, The Netherlands, for testing. From case subjects, tumor biopsy specimens were also collected and frozen immediately at -70°C .

Ethical committees at IARC and in Thailand approved the protocols, and written informed consent was obtained from all subjects. A total of 386 (98%) of the eligible case subjects and 354 (49%) of the eligible control subjects were interviewed. Refusal to participate was the main reason for nonparticipation of control subjects.

The final diagnosis was defined by histologic review of specimens from 350 (91%) case subjects. Because histologic slides were not provided for the remaining 36 (9%), a diagnosis was reached by review of the cytologic materials (n = 7) or by reliance on local histologic diagnosis (n = 29). Cancers in the case subjects were classified as follows: squamous cell carcinoma (n = 345), adenocarcinoma (n = 35), and adenosquamous carcinoma (n = 6). The latter two groups were combined for analysis. Information on stage of the tumors (6) was available for more than 99% (n = 385) of the case subjects, and stage distributions were as follows: 24% stage I, 43% stage II, 27% stage III, and 5% stage IV. Adenocarcinomas/adenosquamous carcinomas tended to be diagnosed at earlier stages; 45% of them were diagnosed at stage I.

On the basis of the degree of nuclear and architectural abnormalities and the proportion of cells showing cytoplasmic differentiation (squamous and glandular), the 355 carcinomas for which evaluable materials were available were graded as follows: well differentiated (n = 150; 42%), moderately well differentiated (n = 131; 37%), and poorly differentiated (n = 74; 21%). Adenocarcinomas/adenosquamous carcinomas tended to be better differentiated;

70% of them were reported to be well differentiated. The presence of inflammatory cells and the number of mitoses per field were also recorded.

For five of the 386 case subjects, we did not have exfoliated cells or biopsy material for determination of HPV; those case subjects were excluded from this analysis. An additional four case subjects were excluded because of inadequate samples (β -globin negative; *see below*). Because one batch was lost during shipment, exfoliated cells were not available for 63 control subjects, who were consequently excluded. An additional 30 control subjects who were β -globin negative and HPV DNA negative were also excluded. Thus, the final group consisted of 377 case subjects (338 squamous cell carcinomas and 39 adenocarcinomas/adenosquamous carcinomas) and 261 control subjects.

Detection of HPV DNA

HPV DNA detection on cervical scrape specimens was performed by PCR (7). To analyze the quality of target DNA for PCR purposes, we pre-screened scrapes by PCR with the use of β -globin gene-specific primers (8). Scrapes that showed successful amplification of β -globin sequences were subjected to HPV DNA genotyping, which was carried out as follows: Briefly, a first screening to determine the overall presence of HPV was performed by use of general primer GP5+/6+-mediated PCR, which permits the detection of a broad spectrum of sequenced and still-unsequenced genital HPV types at the subpicogram level (9). HPV positivity was assessed by low-stringency Southern blot analysis of PCR products with a cocktail probe of HPV-specific DNA fragments (10). Subsequently, GP5+/6+-mediated PCR was performed in triplicate on positive samples to generate sufficient products for further typing. PCR products from independent reactions were pooled to generate identical Southern blots for parallel and successive hybridization rounds for typing with the use of internal type-specific oligoprobes (11). The strategy of melting and rehybridization of the filters was essentially the same as that described earlier (12). The first hybridization round was performed for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, and 56. The second round included HPV types 6, 11, 26, 34, 40, 42, 43, 44, 58, 59, and 68. The third round was performed for HPV types 57, 61, 66, 70 (equivalent to CP141-7), 72 (equivalent to CP4137), 73 (equivalent to MM9), IS39 and MM4 (related to HPV51), MM7, CP6108, and CP8304. The latter three are phylogenetically related to the low-risk HPV61 and HPV62 (8,10). Type-specific oligonucleotides (30-mers) were selected on the basis of sequence information from the HPV sequence database (13,14) after alignment analysis with the CLUSTAL program (PC/Gene, Release 6.7; Intelligenetics, Inc., Mountain View, CA) (15).

Samples that were still GP5+/6+ positive and could not be identified by the above-mentioned probes were considered as HPV X.

In a second step, for those specimens found to be β -globin PCR negative as well as HPV PCR negative, DNA was extracted from the cell pellets and retested for HPV DNA as described above. In addition, for those cervical cancer case subjects from whom not enough cell pellet was available (β -globin negative) as well as for those testing negative for HPV in the exfoliated cells despite an adequate

specimen, the HPV DNA analysis was done on the corresponding biopsy specimens. For HPV DNA detection in the biopsy specimen, the sandwich method (i.e., snap-frozen tissues were cut in a way that the outer sections of a series of sections were used for histologic analysis and the inner sections were used for PCR testing) was used in which the quality of the tissue was controlled as described earlier (16).

We took special precautions to minimize false-positive results in the PCR, as has been described in detail elsewhere (10).

Statistical Analysis

For the estimation of the risk of cervical cancer associated with HPV types and the other variables, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated as approximations of relative risks by use of unconditional logistic regression (17). Adjustment for potential confounders was performed by use of this method. The ORs associated with HPV were adjusted only for age (categorized in four age groups: <40 years, 40-49 years, 50-59 years, and ≥ 60 years) because adjustment for all other relevant risk factors did not materially change the estimates. ORs are presented only for HPV types detected in at least four case subjects. For the analysis of other risk factors, a model including the main variables significantly associated with risk in the univariate model and related to the hypotheses under evaluation was prepared. For the analysis of cofactors of HPV, a model restricted to the HPV-positive case subjects and control subjects was designed; it also included adjustment for all the relevant risk factors.

An initial comparison of case subjects and control subjects revealed that case subjects were more likely than control subjects to be residents of provinces other than the province of the participating hospital, raising the possibility of selection bias. Therefore, the effect of place of residence was examined in the analysis, but it was later removed from the final model because risk estimates changed only minimally when place of residence was included.

HPV attributable fractions (AFs) have been calculated according to standard methods, $AF = p(r - 1) / [1 + p(r - 1)]$, where p = HPV DNA prevalence in the population approximated by the prevalence in the control group and r = relative risk approximated by the OR.

Statistical significance was tested by use of the likelihood ratio test for the difference for dichotomous variables and for two-sided linear trend for variables with logically ordered categories.

Results

The mean age for patients with squamous cell carcinoma was 50.3 years; for patients with adenocarcinoma/adenosquamous carcinoma, it was 46.1 years; for control subjects, it was 49.7 years. In the final group, case subjects and control subjects were distributed similarly by age group, religion, church attendance, and residence in urban versus rural areas.

Overall prevalence of HPV detection among case subjects was 95% for those with squamous cell carcinoma and 90%

for those with adenocarcinoma/adenosquamous carcinoma and did not vary substantially according to demographic characteristics or exposure to different risk factors. Among control subjects, overall HPV prevalence in exfoliated cells was 16% and was associated inversely with age, with a positivity of 20% among women under age 35 that decreased consistently with age to 6% in the group 65 years old or older (P for trend = .13). A slight tendency toward increasing positivity was observed with increasing education (P for trend = .29), but there was no association with age at first intercourse or number of sexual partners, although in this population very few women reported three or more sexual partners, a group in whom the prevalence of HPV detection was 0.

Sixteen different HPV types were de-

tected in specimens of squamous cell carcinoma (Table 1), four were found in specimens of adenocarcinoma/adenosquamous carcinoma, and 10 were found in specimens from control subjects. The most common HPV type in women with squamous cell carcinoma was HPV type 16 followed by HPV types 18, 58, 52, 31, 33, and 59. Among women with adenocarcinoma/adenosquamous carcinoma, HPV type 18 was the most common type followed by types 16 and 45. HPV type 16 was the most common type in control women, followed by types 18, 31, 45, and 58. However, the prevalence of individual types among control subjects was relatively low (e.g., 5% for HPV16 and 3% for HPV18), resulting in very high relative risks. HPV types 34, 40, 44, 51, 54, 56, 57, 61, 66, 68, 72, 73, IS39, MM4, MM7, CP6108, and CP8304 were

not identified in any of the tumor specimens.

Two or more different HPV types were detected in 12 (3.7%) of the specimens of positive squamous cell carcinomas, two (5.7%) of the specimens of positive adenocarcinomas/adenosquamous carcinomas, and four (9.8%) of the specimens of positive control subjects. The majority of instances of double infections included infections with HPV16 or HPV18.

Table 2 presents the ORs for risk of cervical cancer associated with the eight HPV types most commonly detected in this study. For squamous cell carcinoma, strong associations with risk were observed for any HPV (OR = 119), as well as for HPV16 (OR = 227), HPV18 (OR = 115), HPV31 (OR = 44), HPV33 (OR = 70), HPV45 (OR = 20), HPV52 (infinite OR), HPV58 (OR = 66), and HPV59 (infinite OR). The OR for risk of cervical cancer associated with unidentified HPV types was 40. For adenocarcinoma/adenosquamous carcinoma, strong associations with risk were observed for HPV16 (OR = 63), HPV18 (OR = 278), HPV45 (OR = 16), and unknown types (OR = 6.6).

The fraction of risk attributable to HPV in the population was 95% for squamous cell carcinomas and 89% for adenocarcinomas/adenosquamous carcinomas.

Table 3 presents ORs associated with squamous cell carcinoma risk factors other than HPV. In univariate analysis (age-adjusted), the following factors were associated with risk: increasingly limited education, decreasing age at first intercourse, increasing number of live births, number of lifetime sexual partners, history of venereal disease, use of hormonal contraceptives for longer than 4 years, and ever smoking. In addition, a history of previous cytologic screening was associated with a substantial reduction in risk after exclusion of examinations done in the year before diagnosis for the case subjects and date of interview for the control subjects. After adjustment for the presence of HPV DNA in the cervix and for the mutual confounding effect of the other variables, the association with education became not significant (P for trend = .13). The variables that remained associated with risk were number of lifetime sexual partners (P for trend = .003), history of increasing number of episodes of venereal diseases (P for trend = <.001),

Table 1. Human papillomavirus (HPV) types in patients with cervical cancer and control subjects in Thailand*

HPV type	Squamous cell carcinoma		Adenocarcinoma/adenosquamous carcinoma		Control subjects	
	No.	%	No.	%	No.	%†
Negative	16	4.7	4	10.3	220	84.3
Positive for any HPV type	322	95.3	35	89.7	41	15.7
Total	338	100.0	39	100.0	261	100.0
HPV16	186	57.8	12	34.3	10	24.4
HPV18	52	16.1	19	54.3	6	14.6
HPV26	1	0.3				
HPV31	5	1.6			3	7.3
HPV33	5	1.6				
HPV35	1	0.3				
HPV39	2	0.6				
HPV40					1	2.4
HPV43	1	0.3			1	2.4
HPV45	3	0.9	1	2.9	2	4.9
HPV52	7	2.2				
HPV58	9	2.8			2	4.9
HPV59	5	1.6				
HPV70	2	0.6			1	2.4
HPV X	31	9.6	1	2.9	11	26.8
HPV6 + HPV58	1	0.3				
HPV16 + HPV6	1	0.3				
HPV16 + HPV11	1	0.3				
HPV16 + HPV18	3	0.9	1	2.9	1	2.4
HPV16 + HPV31	1	0.3				
HPV16 + HPV42	1	0.3				
HPV16 + HPV35					1	2.4
HPV18 + HPV52	2	0.6				
HPV18 + HPV59			1	2.9		
HPV31 + HPV45	1	0.3				
HPV33 + HPV45					1	2.4
HPV35 + HPV45	1	0.3				
HPV45 + HPV70					1	2.4

*Based on results from exfoliated cells and biopsy specimens in case subjects and exfoliated cells only in control subjects. Percentage of HPV types are calculated relative to the total HPV positive and indicates percentage of positive cases in which a specific type alone or in combination was detected. The total percentage of a specific HPV type requires the addition of case subjects in which it appears in combination with other types.

†Beginning with the fourth value in this column (i.e., 24.4), all percentages reflect the percent of HPV-positive control subjects.

Table 2. Prevalence of type-specific HPV DNA detection and age-adjusted odds ratios (ORs) for association between adenocarcinoma/adenosquamous carcinoma and squamous cell carcinoma of the cervix and HPV types

HPV type	Case subjects						OR (95% confidence interval)	
	Squamous cell carcinoma		Adenocarcinoma/adenosquamous carcinoma		Control subjects		Squamous cell carcinoma	Adenocarcinoma/adenosquamous carcinoma
	No.	HPV prevalence, %	No.	HPV prevalence, %	No.	HPV prevalence, %		
Negative.....	16	4.7	4	10.3	220	84.3	1.0 (referent)	1.0 (referent)
Positive for any HPV type.....	322	95.3	35	89.7	41	15.7	119 (64-222)	53 (17-163)
Total.....	338	100.0	39	100.0	261	100.0		
HPV16	193	57.1	13	33.3	12	4.6	227 (103-497)	63 (17-232)
HPV18	57	16.9	21	53.8	7	2.7	115 (45-299)	278 (50-1535)
HPV31	7	2.1			3	1.1	44 (9-221)	
HPV33	5	1.5			1	0.4	70 (7-696)	
HPV45	5	1.5	1	2.6	4	1.5	20 (5-89)	16 (1.2-228)
HPV52	9	2.7			0	0.0	∞ (31-∞)	
HPV58	10	3.0			2	0.8	66 (13-335)	
HPV59	5	1.5			0	0.0	∞ (15-∞)	
HPV X	31	9.2	1	2.6	11	4.2	40 (17-97)	6.6 (0.6-71)
Other types	12	3.6	1	2.6	5	1.9	51 (13-206)	5.7 (0.5-69)
Multiple types*	12	3.6	2	5.1	4	1.5	49 (13-184)	30 (3-280)

*Included in the calculation of prevalence and ORs for individual types.

and interval since last Pap smear (P for trend = .005). When compared with women reporting only one lifetime sexual partner, those reporting two had a 2.5-fold and those reporting three or more had a sevenfold increase in risk. Women who reported having had any venereal disease once had a 2.2-fold increase in risk compared with women who reported never having had venereal diseases, and those who reported more than one episode of venereal disease had a 4.7-fold increase in risk. All the venereal diseases reported (syphilis, gonorrhea, genital warts, herpes genitalis, parasites, and "other") were more common in case subjects than in control subjects, but the most frequently reported was parasites. When compared with women who were never screened, those who were screened 5 or more years before the interview had a 40% reduction in risk, but those who were screened in the previous 5 years had a 70% reduction in risk. When this analysis was restricted to the HPV-positive case subjects and control subjects, the same associations persisted, but smoking again appeared to be associated with a 2.8-fold increase in risk of squamous cell carcinoma. However, no clear evidence of a dose-response effect with amount or duration of smoking was evident.

Table 4 presents risk factors other than HPV for adenocarcinoma/adenosquamous carcinoma. A very similar pattern of association with the different risk factors

was observed, with increases in risk remaining after adjustment for limited education, number of sexual partners, histories of venereal diseases, and some effect of smoking. None of the trends in the adjusted model were, however, statistically significant given the relatively small numbers. In the HPV-positive strata, similar magnitudes of risk were detected for number of sexual partners and histories of venereal diseases, but no associations were observed with smoking.

Discussion

The results of this case-control study conducted in Thailand confirm the findings of other investigators (1) indicating that HPV DNA is present in the vast majority of cervical cancers, regardless of histology. In this investigation, HPV DNA was detected in 95% of squamous cell carcinomas and 90% of adenocarcinomas/adenosquamous carcinomas. This high prevalence of detection of HPV in cervical tumors, now frequently observed in studies applying PCR methods for detection of HPV, casts doubt on the existence of HPV-negative cervical cancer (18).

There are several possible explanations for why some case subjects were scored HPV DNA negative. For example, the biopsy specimen may not have been representative of the tumor tissue or the HPV DNA could have been interrupted by in-

tegration in the host cell DNA, thereby deleting the L1 region that was the target of the PCR used. Other possibilities are the existence of still new HPV types or the presence of HPV types amplifying less efficiently in low copy number (e.g., HPV39 or HPV52).

To ensure that the biopsy specimens on which the PCR was performed were representative of the tumor tissue, we used the sandwich method of sample preparation (16), in which histologic verification of the specimens is carried out. In fact, in some HPV DNA-negative case subjects, the histology was poor and the diagnosis of cancer could not be confirmed in the frozen specimens.

The PCR method that we used is considered highly sensitive (10). Moreover, the strategy used for typing has been tested before (12), indicating that three rounds of melting and rehybridization of the filters do not affect the sensitivity of the assay. Therefore, eventual underrepresentations of HPV types tested in this study are unlikely to be the result of the typing methodology.

Our results with respect to prevalence of HPV by age in the control group agree with previous observations (1) of declining prevalence of infection with increasing age. In this study, the decline did not appear to be as dramatic in the younger ages as has been observed in another study (19), but prevalence declined from approximately 20% among women under

Table 3. Risk factors for squamous cell carcinoma of the cervix in Thailand

Risk factor	All women			Human papillomavirus-positive women
	Case subjects	Control subjects	Odds ratio* (95% confidence interval)	Odds ratio† (95% confidence interval)
Education				Odds ratio‡ (95% confidence interval)
Higher	27	51	1.0 (referent)	1.0 (referent)
Primary	234	160	2.9 (1.7–4.9)	2.4 (0.8–7.3)
None	77	50	3.2 (1.7–6.2)	2.4 (0.5–11.8)
Age at first intercourse, y				
≥24	31	71	1.0 (referent)	1.0 (referent)
21–23	51	55	2.1 (1.2–3.8)	0.9 (0.3–3.4)
18–20	141	81	4.0 (2.4–6.7)	2.8 (0.8–10.5)
<18	115	52	5.1 (3.0–8.7)	1.6 (0.4–6.3)
No. of live births				
0–1	20	30	1.0 (referent)	1.0 (referent)
2–3	85	91	1.4 (0.7–2.6)	0.8 (0.2–4.2)
4–5	106	60	2.8 (1.4–5.4)	1.1 (0.2–6.9)
≥6	127	80	2.7 (1.4–5.3)	0.4 (0.1–2.9)
Lifetime No. of sexual partners				
1	206	211	1.0 (referent)	1.0 (referent)
2	94	41	2.4 (1.6–3.6)	3.4§ (1.1–10.1)
3	38	7	5.5 (2.4–12.7)	
Any venereal disease				
Never	127	152	1.0 (referent)	1.0 (referent)
Once	91	64	1.8 (1.2–2.7)	1.9 (0.7–5.0)
More than once	120	45	3.5 (2.3–5.3)	4.2 (1.4–12.8)
Use of hormonal contraceptives, y¶				
Never	230	189	1.0 (referent)	1.0 (referent)
1–3	51	49	1.0 (0.6–1.5)	0.5 (0.2–1.3)
≥4	57	23	2.2 (1.3–3.8)	2.4 (0.6–9.1)
Smoking status				
Never	186	189	1.0 (referent)	1.0 (referent)
Ever	152	72	2.3 (1.6–3.4)	2.8 (1.0–7.6)
Interval since last Pap smear, y				
Never	255	157	1.0 (referent)	1.0 (referent)
≥5	14	18	0.5 (0.2–0.9)	3.4 (0.2–46.5)
<5	53	69	0.4 (0.3–0.7)	0.4 (0.1–1.1)

*Adjusted for age.

†Adjusted for detection of human papillomavirus (HPV) DNA and all variables shown.

‡Restricted to HPV-positive case subjects and control subjects, adjusted for all variables shown.

§Indicates two or more partners.

||Includes syphilis, gonorrhea, genital warts, herpes genitalis, parasites, and "other."

¶Includes oral and injectable contraceptives.

35 years of age to approximately 6% among women older than 65 years.

HPV16 was the most common HPV type in squamous cell carcinoma (60% of the positives), followed by HPV18 (18%). The overall OR for cervical cancer associated with HPV positivity by any HPV type was 119, and strong associations with risk of squamous cell carcinoma were observed for the eight most common HPV types found in this population (types 16, 18, 31, 33, 45, 52, 58, and 59). In adenocarcinomas/adenosquamous carcinomas, HPV18 was the most common type found (60% of the positives), followed by HPV16 (37%). A more limited number of HPV types was found for this histologic type. Apart from very strong

associations with HPV16 and HPV18, the only other type associated with increased risk of adenocarcinoma/adenosquamous carcinoma of the cervix was HPV45.

Although several case series that used PCR-based assays have reported HPV DNA in substantial proportions of adenocarcinomas (1), to our knowledge, this is one of the first case-control studies (20) in which the risk linked to specific HPV types has been estimated for adenocarcinoma/adenosquamous carcinoma. Our results confirm that, as was found for squamous cell carcinoma, HPV types 18, 16, and 45 (in order of strength of association) are the main causes of adenocarcinoma/adenosquamous carcinoma.

HPV positivity among case subjects

was different when only exfoliated cells or exfoliated cells and biopsy specimens were evaluated (83% versus 95% in squamous cell carcinomas and 84% versus 90% in adenocarcinomas/adenosquamous carcinomas), and from control subjects we had results only from the analysis of exfoliated cells. Thus, the ORs that we present are higher than those obtained when only exfoliated cells from both case and control subjects are tested. However, we consider this to be a valid approach because we have conducted extensive retesting of specimens in our large series of patients with invasive cancer who were from 22 countries (2), and preliminary unpublished results (Walboomers JMM, Jacobs MV, Manos MM, Muñoz N, Bosch

Table 4. Risk factors for adenocarcinoma/adenosquamous carcinoma of the cervix in Thailand

Risk factor	All women				Human papillomavirus-positive women
	Case subjects	Control subjects	Odds ratio* (95% confidence interval)	Odds ratio† (95% confidence interval)	Odds ratio‡ (95% confidence interval)
Education					
Higher	5	51	1.0 (referent)	1.0 (referent)	1.0 (referent)
Primary	27	160	2.3 (0.8–6.5)	3.5 (0.7–18.7)	1.5 (0.2–10.2)
None	7	50	3.6 (0.9–14.2)	5.1 (0.5–52.2)	3.6 (0.3–46.3)
Age at first intercourse, y					
≥24	4	71	1.0 (referent)	1.0 (referent)	1.0 (referent)
21–23	5	55	1.7 (0.4–6.8)	0.3 (0.03–2.5)	0.3 (0.1–5.5)
18–20	16	81	4.1 (1.3–13.1)	1.5 (0.2–10.8)	1.0 (0.1–16.7)
<18	14	52	6.2 (1.9–20.6)	1.7 (0.2–14.4)	0.9 (0.1–14.7)
No. of live births					
0–1	3	30	1.0 (referent)	1.0 (referent)	1.0 (referent)
2–3	15	91	1.4 (0.4–5.3)	0.5 (0.1–6.2)	4.0 (0.2–86.2)
4–5	9	60	1.6 (0.4–6.5)	0.5 (0.1–5.2)	0.7 (0.1–17.3)
≥6	12	80	2.7 (0.6–11.6)	1.1 (0.1–17.7)	4.4 (0.1–161.4)
Lifetime No. of sexual partners					
1	25	211	1.0 (referent)	1.0 (referent)	1.0 (referent)
2	10	41	2.3 (1.0–5.3)	1.5 (0.3–7.0)	5.2§ (0.8–34.9)
≥3	4	7	4.9 (1.2–18.9)	5.8 (0.1–280.0)	
Any venereal disease 					
Never	12	152	1.0 (referent)	1.0 (referent)	1.0 (referent)
Once	14	64	2.4 (1.0–5.7)	3.5 (0.7–18.3)	3.1 (0.4–24.2)
More than once	13	45	3.3 (1.3–8.2)	6.5 (1.1–36.3)	6.4 (0.8–49.9)
Use of hormonal contraceptives, y¶					
Never	23	189	1.0 (referent)	1.0 (referent)	1.0 (referent)
1–3	7	49	1.0 (0.4–2.7)	0.7 (0.1–3.5)	0.4 (0.1–3.2)
≥4	9	23	2.5 (1.0–6.5)	2.7 (0.5–15.5)	2.2 (0.2–22.5)
Smoking status					
Never	26	189	1.0 (referent)	1.0 (referent)	1.0 (referent)
Ever	13	72	2.8 (1.2–6.7)	1.9 (0.4–8.9)	1.0 (0.2–6.8)
Interval since last Pap smear, y					
Never	22	157	1.0 (referent)	1.0 (referent)	1.0 (referent)
≥5	7	18	2.4 (0.8–6.7)	1.8 (0.2–13.4)	6.0 (0.2–189.0)
<5	7	69	0.5 (0.2–1.2)	0.5 (0.1–2.3)	0.3 (0.1–2.4)

*Adjusted for age.

†Adjusted for detection of human papillomavirus (HPV) DNA and all variables shown.

‡Restricted to HPV-positive case subjects and control subjects, adjusted for all variables shown.

§Indicates two or more partners.

||Includes syphilis, gonorrhea, genital warts, herpes genitalis, parasites, and "other."

¶Includes oral and injectable contraceptives.

FX, Kummer A) indicate that HPV is detectable in almost 99% of the case subjects. In this context, we observed in this study that undifferentiated tumors and tumors with high number of mitoses had significantly lower HPV detection rates when exfoliated cells were analyzed than when results from cells and biopsy specimens (74% versus 94%) were included, whereas well-differentiated tumors with few mitoses had similar prevalence (90% versus 94%) (data not shown), indicating that the more undifferentiated cancers may have a limited ability to exfoliate (i.e., detach and shed superficial cells) cells with detectable HPV DNA. This association of HPV positivity with degree of differentiation could be an indirect in-

dication that, among control subjects, exfoliated cells may suffice to provide a reliable estimate of the presence of HPV DNA. To resolve the issue of possible bias in the ORs calculated, we are conducting a sampling validation study in which cells and biopsy specimens have been collected from normal cervixes of women undergoing hysterectomy for reasons other than cancer. Preliminary analysis of this study indicates that the prevalence of HPV positivity is almost identical when exfoliated cell results are used to that obtained with biopsy specimens (data not shown).

Investigation of cofactors in this analysis revealed statistically significant associations with the number of sexual part-

ners and histories of venereal disease as well as an association with smoking that was restricted to squamous cell carcinomas. These associations were evident even when the analysis was restricted to HPV-positive women, which practically eliminated the possibility of this being the result of residual confounding by HPV. The association with increasing number of sexual partners may be related to transmission in this population of other sexually transmitted agents that can act as cofactors of HPV. This hypothesis is supported by the high frequency of reported histories of venereal diseases among both case subjects and control subjects and its strong association with risk. Without additional data, it is hard to point

to a specific sexually transmitted agent, since most of this association was driven by the effect of parasites, which is obviously an indirect marker. However, all other sexually transmitted diseases were also associated with risk. We are currently investigating the role of chlamydia and herpes simplex virus type II in serologic specimens in this population. A recently published case-control study in Thailand (21) indicated a high prevalence of histories of visits to prostitutes among Thai males. It is of interest that, in our study, risk factors were similar for squamous cell carcinomas and adenocarcinomas/adenosquamous carcinomas, with the exception of smoking, which appears to play a role only in squamous cell carcinomas. On the other hand, the associations with age at first intercourse, number of live births, and use of hormonal contraceptives disappeared after adjustment. The results of other published case-control studies of invasive cancer (22-24), including our companion report (20), have found strong, independent associations with some of these variables, but the pattern is not consistent, indicating the possibility of different cofactors playing a role in different areas and the need for further research.

Potential limitations should be considered in the interpretation of our results. Our study was hospital based; as such, it may suffer from selection bias. The case subjects were consecutive incident case subjects attending the largest hospital in Hat-Yai, and most of them agreed to participate. The control subjects were attending the same hospital, and it can be assumed that they came from the same population as the case subjects. The fact that all case subjects were hospitalized with a variety of diseases, some of which were infectious, could theoretically increase the prevalence of HPV in the control group if some had an impaired immune status. However, this would only underestimate our relative risks associated with HPV. The refusal rate for control subjects was very high (50%), and 60 control specimens were lost during shipment. In addition, 30 control specimens had to be excluded because they were β -globin negative. Given this important loss of control subjects, we cannot exclude the possibility of selection bias. However, the prevalence of HPV in the control group was higher than that reported in other

studies, suggesting that, if there is selection bias, it could have led to an underestimation of the relative risk associated with HPV. On the other hand, the final group of control subjects was similar to the case subjects in age, ethnicity, and religion, suggesting a nondifferential loss of control subjects. Another potential limitation is that we had small numbers of case subjects with some of the HPV types, which can add uncertainty to the magnitude of risk associated with some of the unusual HPV types.

In summary, HPV DNA appears to play a carcinogenic role in almost all squamous cell carcinomas and adenocarcinomas/adenosquamous carcinomas of the cervix in Thailand, as was found elsewhere. HPV types 16, 18, 31, 33, 45, 52, 58, and 59 are particularly involved in the etiology of squamous cell carcinomas, and HPV types 16, 18, and 45 are associated with adenocarcinomas/adenosquamous carcinomas. The fact that a limited number of HPV types are emerging as responsible for the majority of cervical cancers in most geographic areas will facilitate the introduction of globally useful control strategies, based on HPV vaccines. In this population, in particular, other yet unidentified sexually transmitted agents are likely cofactors of HPV.

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Notes

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Folate Intake, Alcohol Consumption, Cigarette Smoking, and Risk of Colorectal Adenomas

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Background: Recent evidence suggests that folic acid (and derivatives) could contribute to the protective effect of fruits and vegetables against the risk of large-bowel cancer. Other evidence indicates that alcohol drinking and cigarette smoking may impair the biologic actions of folate. We used data from an adenoma prevention trial to investigate the occurrence of colorectal adenomas (possible precursors of colorectal cancer) in association with folate intake, alcohol consumption, and cigarette smoking. **Methods:** Patients with at least one recent large-bowel adenoma were followed with colonoscopy 1 year and 4 years after their qualifying colon examinations. Adenomas detected after the year 1 examination were used as end points. A food-frequency questionnaire was administered at study entry and at study completion; nutrient intake at study entry was used in this analysis. All statistical tests were two-sided. **Results:** After adjustment for caloric intake, dietary folate had a significant protective association with the risk of recurrence of large-bowel adenoma (P for trend = .04). However, this inverse association was attenuated by further adjustment for intake of dietary fiber and fat. Use of folate supplements was not associated with a reduction in risk. Alcohol intake (seven or more drinks/week) was associated with increased risk (odds ratio = 2.04; 95% confidence interval = 1.28-3.26). Cigarette smoking, even smoking for long duration, was not related to adenoma recurrence. **Conclusions:** These data provide only modest support for previous findings suggesting beneficial effects of folate on colorectal adenoma risk. We find no evidence that cigarette

smoking increases risk. These findings do suggest a substantial increase in risk with alcohol consumption. [*J Natl Cancer Inst* 1998;90:57-62]

An apparent protective effect of fruits and vegetables on the risk of cancer of the large bowel has been a consistent epidemiologic finding, but the food constituents that may have an anticarcinogenic effect have not been clearly identified (1). Recent investigation has suggested that folate (folic acid and its derivatives such as tetrahydrofolate and 5-methyl-tetrahydrofolate) could contribute to such a protective effect. Folate-deficient rats demonstrate heightened sensitivity to carcinogens (2), and some epidemiologic studies (3-9) have reported that individuals with high folate intake have a reduced risk of colorectal cancer or colorectal adenomas. The mechanism by which folate might exert an anticarcinogenic effect is not clear but may relate to its role in DNA methylation (10).

Cigarette smoking and alcohol intake are two common habits that may impair the biologic actions of folate. Ethanol and its metabolites appear to reduce circulating folate levels and to interfere with some of its biochemical actions (7). Alcohol intake has been associated with an increased risk of colorectal cancer (11,12). However, the results obtained from investigations on this point have not been consistent, and most epidemiologic studies have not shown a strong relationship (11,12). In contrast, alcohol intake is more clearly associated with the risk of large-bowel adenomas (13).

Cigarette smoking also appears to have an antifolate effect. Smoking is associated with decreased circulating folate levels

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(14,15), and an antagonism of folate by constituents of cigarette smoke has been hypothesized (16,17). Most studies have not found an association between cigarette smoking and large-bowel cancer (18,19), although there is inconsistent evidence of an increased risk from long-term smoking or after a lengthy latent period (19–22). Several investigations of adenoma risk have reported an association with smoking, although others have reported no association [reviewed in (13)]. Thus, the role of folate intake and folate-depleting exposures remains uncertain.

We report here on the relationship between folate intake, alcohol consumption, and cigarette smoking and adenoma risk among participants in an adenoma prevention clinical trial.

Subjects and Methods

Study subjects were participants in a multicentered clinical trial of β -carotene (25 mg daily) and a combination of vitamin C (1 g daily) and vitamin E (400 mg daily) as preventive agents against recurrence of large-bowel adenomas. The main study results have been published; antioxidant supplementation did not affect the risk of adenoma recurrence (23). Local human subjects review committees approved the protocol at each study site. Each study subject provided informed written consent and had at least one large-bowel adenoma excised within the 3 months before study entry, with no known polyps left in the bowel. Patients with familial polyposis, a history of invasive colorectal cancer, or malabsorption syndromes were excluded, as were subjects with conditions that might be worsened by vitamin C or E, such as renal calculi or thrombophlebitis, and those unwilling to forego supplements containing study agents. Colonoscopic follow-up was scheduled at 1 year and at 4 years after the qualifying colonoscopic examination. Adenomas detected after the 1-year examination up to and including the 4-year examination were the principal end points of the study. Of the 864 subjects originally randomly assigned in the trial, 44 died before the 4-year examination, 32 no longer wished to participate in the study, 19 could not be examined because they had moved or were too ill, and 18 did not complete the final colonoscopy for unknown reasons, leaving 751 subjects with complete follow-up information.

At study entry and at the end of the trial, subjects completed a semiquantitative food-frequency questionnaire requesting information regarding dietary intake during the prior year. This validated instrument included 100 food items (plus open-ended questions for frequently eaten but unlisted foods). Using DIETSYS (24), the database and programs developed at the National Cancer Institute, we estimated the intake of the most commonly analyzed nutrients. The folate content of selected food items was taken from the DIETSYS database (24). The principal analyses utilized intake estimated from the baseline dietary assessment, i.e., before the risk period of the trial. Secondary analyses used data from

the questionnaire administered at exit from the study.

Cigarette smoking status was assessed at entry into the study through a questionnaire regarding current number of cigarettes smoked daily and the age of initiation of smoking and (if a former smoker) the age at smoking cessation and the number of cigarettes formerly smoked. Alcohol intake (as beer, wine, or spirits) was measured at study entry as part of the beverage portion of the dietary questionnaire.

We assessed use of vitamin (including folate) and mineral supplements and over-the-counter drugs from responses to the baseline questionnaire and to interval questionnaires (i.e., questionnaires mailed to subjects every 6 months). The proportion of interval questionnaires in which a particular supplement was reported as being used was calculated as a summary index of use of the product during the trial. "Total folate" intake was taken to be the sum of intakes from dietary and supplemental sources.

We used odds ratios (ORs) and 95% confidence intervals (CIs) of having at least one recurrent adenoma as measures of association with an exposure; these were calculated by use of unconditional logistic regression. To adjust for possible confounding from total energy intake, we entered nutrients into the models as residuals from the regression of the logarithm of the nutrient on the logarithm of total calories (25). Multivariate models are presented, with adjustment for age, sex, clinical center, and the interval between 1-year and 4-year colonoscopic examinations (basic "demographic" covariates). In more detailed models, intakes of total calories, dietary fat, and dietary fiber were also added as covariates. Further adjustment for randomized treat-

ment assignment had virtually no effect; these estimates are not shown. In analyses that considered different regions of the large bowel, the right bowel was taken to include the cecum, the ascending colon, and the transverse colon; the left bowel included the more distal portions of the bowel. All statistical tests were two-sided.

Results

Of the 751 subjects who had both 1-year and 4-year colonoscopies, 709 satisfactorily completed the baseline dietary questionnaire and were included in the analysis presented here. Participants were predominantly male, with a mean age of about 60 years (Table 1). Subjects who were excluded from the analysis because of death, loss to follow-up, or lack of dietary data were similar to those who were included with regard to age, sex, total caloric intake, and intake of dietary fat. However, subjects who were not included tended to drink less alcohol, smoke more cigarettes, and report lower intakes of dietary folate and total dietary fiber (data not shown).

At study entry, 64% of subjects reported use of folate supplements (largely from multivitamin preparations), but because of the restrictions on these prepara-

Table 1. Characteristics of subjects participating in the study

	Adenoma recurrence	No adenoma recurrence
No. of subjects	260	449
Males, %	81.5	76.6
Mean age, y*	61.9 \pm 8.3	60.7 \pm 8.3
Ever smokers, %	50.8	45.9
Current smokers at study entry, %	17.7	18.9
Mean alcohol intake, drinks/week*	1.1 \pm 1.8	0.7 \pm 1.6
Mean caloric intake, kcal/day*	1996 \pm 727	1954 \pm 776
Mean total dietary fiber intake, g/day*	13.8 \pm 6.8	14.8 \pm 7.5
Mean dietary folate intake, μ g/day*	311.7 \pm 9.2	323.4 \pm 8.3
Mean intake of foods, servings/week*		
Broccoli, cauliflower, and Brussels sprouts	1.2 \pm 1.4	1.6 \pm 2.2
Spinach	0.5 \pm 0.7	0.5 \pm 1.2
Orange juice	3.7 \pm 6.2	3.2 \pm 5.1
Oranges	1.5 \pm 3.6	1.6 \pm 3.4
Potatoes	3.8 \pm 4.1	3.9 \pm 4.1
Green salad	3.3 \pm 4.4	3.3 \pm 4.7
Dried beans	0.6 \pm 0.7	0.8 \pm 1.7
Peas and green beans	2.1 \pm 1.7	1.9 \pm 1.7
High-fiber and nonfortified cold cereals	3.4 \pm 6.1	3.7 \pm 6.4
Dark bread	4.2 \pm 6.1	3.9 \pm 5.8
Eggs	1.9 \pm 3.8	1.5 \pm 1.8
Liver	0.2 \pm 0.3	0.1 \pm 0.2
% of participants reporting supplementary folate use		
At study entry	66.0	62.9
After randomization	9.2	13.1

*Values = means \pm standard deviation.

Table 2. Baseline folate intake and adenoma recurrence risk*

Folate intake	No. with recurrence/ No. without recurrence	Entire colorectum		Right colorectum: multiply adjusted† OR (95% CI)	Left colorectum: multiply adjusted† OR (95% CI)
		Calorie-adjusted OR (95% CI)	Multiply adjusted† OR (95% CI)		
Dietary folate‡					
Quartile I	69/103	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)
Quartile II	75/113	0.89 (0.57–1.38)	1.06 (0.67–1.70)	1.12 (0.66–1.90)	0.92 (0.54–1.57)
Quartile III	57/115	0.66 (0.42–1.04)	0.82 (0.50–1.35)	0.78 (0.43–1.38)	0.83 (0.47–1.47)
Quartile IV	59/118	0.65 (0.41–1.04)	0.94 (0.53–1.67)	1.00 (0.52–1.92)	0.73 (0.38–1.43)
<i>P</i> for trend		.04	.69	.78	.54
Total folate§					
Quartile I	65/117	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)
Quartile II	69/109	1.07 (0.68–1.67)	1.08 (0.69–1.70)	1.12 (0.66–1.90)	0.85 (0.50–1.42)
Quartile III	64/111	0.90 (0.57–1.41)	0.97 (0.61–1.52)	1.30 (0.77–2.19)	0.72 (0.42–1.23)
Quartile IV	61/110	0.90 (0.57–1.41)	1.11 (0.69–1.78)	1.11 (0.63–1.97)	0.97 (0.56–1.67)
<i>P</i> for trend		.73	.57	.41	.90

*OR = odds ratio; CI = confidence interval.

†Adjusted for age, sex, clinical center, fat residuals, total dietary fiber residuals, energy intake, and colonoscopy interval.

‡Median calorie-adjusted intakes in quartiles of dietary folate were 214, 269, 309, and 388 µg/day.

§Median calorie-adjusted intakes in quartiles of total folate were 243, 338, 366, and 391 µg/day. Three subjects were excluded because supplement information was missing for them.

Table 3. Intake of folate-containing foods at baseline and colorectal adenoma risk: highest versus lowest quartile of intake*

	Folate content, µg/medium serving	Calorie-adjusted OR (95% CI), quartile IV versus quartile I	Calorie-, fat-, and fiber-adjusted OR (95% CI), quartile IV versus quartile I
Broccoli, cauliflower, and Brussel sprouts	52	1.21 (0.71–2.08)	1.44 (0.81–2.56)
Spinach	91	1.17 (0.73–1.86)	1.31 (0.80–2.13)
Orange juice	81	1.03 (0.63–1.69)	1.04 (0.63–1.73)
Oranges	30	0.73 (0.46–1.15)	0.84 (0.52–1.35)
Potatoes	8	0.51 (0.29–0.87)	0.45 (0.26–0.78)
Green salad	43	1.12 (0.73–1.72)	1.42 (0.88–2.29)
Dried beans	117	1.07 (0.68–1.68)	1.09 (0.69–1.73)
Peas and green beans	29	1.10 (0.73–1.67)	1.24 (0.79–1.92)
Fortified cereals†	395	0.89 (0.58–1.37)	0.95 (0.61–1.48)
High-fiber-containing and other cold cereals	110	0.54 (0.33–0.88)	0.62 (0.37–1.05)
Dark bread	29	0.64 (0.38–1.08)	0.73 (0.42–1.24)
Eggs	47	1.77 (1.10–2.85)	1.64 (1.00–2.69)
Liver†	187	1.38 (0.87–2.21)	1.40 (0.87–2.24)

*All models were adjusted for age, sex, clinical center, and colonoscopy interval. ORs are calculated per serving/week. OR = odds ratio; CI = confidence interval.

†Highest versus lowest tertile.

tions imposed by the trial, fewer than 15% took any supplemental folate during the trial. Estimated dietary folate intake (mean ± standard error) remained relatively constant during the study: 319 ± 6.2 µg/day at study entry and 336 ± 5.9 µg/day at study completion.

Effect of Folate Intake

After adjustment for caloric intake, dietary folate intake was inversely associated with the risk of adenoma recurrence (Table 2). Compared with subjects in the lowest quartile of intake, those in the highest quartile had a calorie-adjusted OR of 0.65 (95% CI = 0.41–1.04), and those

in the highest decile had an OR of 0.57 (95% CI = 0.31–1.05). Findings were similar in the right and left bowel (Table 2) and among men and women (data not shown). Adjustment for dietary fat and total dietary fiber considerably attenuated the folate–adenoma association (Table 2). Total folate intake was essentially unrelated to adenoma recurrence (Table 2).

Some folate-containing foods (e.g., oranges, potatoes, dark bread, and breakfast cereals) were inversely related to adenoma risk (Table 3). However, intake of other folate-rich foods (beans, broccoli–cauliflower–Brussels sprouts, green salad, eggs, and liver) was not associated with a

decreased risk (Table 3). Additional adjustment for aspirin intake did not alter these folate-related estimates, and ORs obtained by use of the food-frequency questionnaire administered at study exit were similar to those presented (data not shown).

Use of folate supplements of any duration after randomization conferred an OR versus no use of 0.75 (95% CI = 0.45–1.26; *P* for trend over the proportion of interval questionnaires reporting use = .40). Further adjustment for dietary factors did not materially alter these estimates (data not shown). Supplement use at study entry was unrelated to recurrence risk (data not shown).

Effects of Alcohol Consumption and Cigarette Smoking

Alcohol consumption had a threshold effect on the risk of adenoma recurrence. Fewer than seven alcoholic drinks/week had no effect, but the diet-adjusted OR rose to 2.04 (95% CI = 1.28–3.26) for seven or more alcoholic drinks/week in comparison to nondrinkers. There was no further increase in risk among those taking 14 or more drinks/week (data not shown). Results were similar for the right and left bowel (Table 4) and for men and women (data not shown). There were no substantial differences in relative risk estimates according to the different types of alcoholic beverages consumed (data not shown). Alcohol intake data from the questionnaire at study exit also suggested

Table 4. Baseline alcohol intake and cigarette smoking and colorectal adenoma risk*

	No. with recurrence/ No. without recurrence	Entire colorectum, OR (95% CI)	Right colorectum, OR (95% CI)	Left colorectum, OR (95% CI)
Alcohol intake[†]				
None	68/134	1.00 (referent)	1.00 (referent)	1.00 (referent)
<1 drink/week	35/79	0.96 (0.58–1.60)	1.50 (0.82–1.72)	0.75 (0.41–1.38)
1–6 drinks/week	61/133	0.95 (0.61–1.47)	1.51 (0.90–2.54)	0.62 (0.37–1.06)
≥7 drinks/week	96/101	2.04 (1.28–3.26)	2.09 (1.20–3.60)	1.67 (1.00–2.80)
Smoking status				
Never smoker	82/158	1.00 (referent)	1.00 (referent)	1.00 (referent)
Former smoker	132/206	1.12 (0.78–1.61)	0.95 (0.62–1.44)	1.36 (0.88–2.09)
Current smoker	46/85	0.95 (0.59–1.52)	0.89 (0.51–1.53)	1.44 (0.84–2.49)
Amount smoked, cigarettes/day[‡]				
Never smoker	82/158	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20	85/121	1.20 (0.81–1.80)	0.96 (0.60–1.53)	1.45 (0.91–2.31)
21–40	55/123	0.78 (0.50–1.22)	0.72 (0.43–1.21)	1.23 (0.74–2.06)
>40	35/41	1.55 (0.89–2.72)	1.35 (0.73–2.51)	1.71 (0.90–3.26)
Duration smoker, y[§]				
Never smoker	82/158	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20	52/68	1.52 (0.94–2.45)	1.06 (0.61–1.85)	1.96 (1.14–3.37)
>20, ≤30	30/69	0.71 (0.42–1.21)	0.51 (0.26–0.99)	1.15 (0.63–2.10)
>30, ≤40	48/77	1.05 (0.66–1.68)	1.26 (0.75–2.11)	0.92 (0.51–1.64)
>40	41/69	0.97 (0.59–1.61)	0.80 (0.45–1.43)	1.66 (0.94–2.94)

*Adjusted for age, sex, clinical center, fat residuals, total dietary fiber residuals, energy intake, and colonoscopy interval. OR = odds ratio; CI = confidence interval.

[†]Two patients with missing information are excluded.

[‡]Nine patients with missing information are excluded.

[§]Fifteen patients with missing information are excluded.

an increased risk only with consumption of seven or more drinks/week, but with a less marked increase in risk (OR versus nondrinkers = 1.30; 95% CI = 0.83–2.04).

Cigarette smoking was not associated with adenoma recurrence (Table 4); current smokers had a diet-adjusted OR of 0.95 (95% CI = 0.59–1.52), and former smokers had a diet-adjusted OR of 1.12 (95% CI = 0.78–1.61). Female current smokers had a low adenoma risk, which, however, was compatible with chance (OR = 0.38; 95% CI = 0.11–1.27), after adjustment for demographic and nutritional variables. The difference in relative risk for smoking status between men and women was not statistically significant (P for interaction = .22).

There was no indication that heavy smoking or long-term smoking substantially altered risk. Subjects who ever smoked more than 40 cigarettes/day had a multivariate-adjusted OR of 1.55 (95% CI = 0.89–2.72); smoking for more than 40 years was associated with an OR of 0.97 (95% CI = 0.59–1.61). Even among current smokers, there was no suggestion of an increased risk among heavy smokers or long-term smokers (data not shown).

Joint Effect of Alcohol and Folate Intake

We assessed adenoma risk in potentially “high-risk” individuals who had a calorie-adjusted folate intake below the median level and alcohol intake above the median level. “Low-risk” subjects had the converse: median folate intake above and alcohol intake below their respective median levels. The remaining subjects constituted the “intermediate” group. The potentially high risk subjects exhibited a substantially increased adenoma risk compared with the risk for the intermediate group (diet-adjusted OR = 1.85; 95% CI = 1.15–2.97), but the potentially low risk subjects did not experience a lower risk in comparison to those subjects (OR = 0.99; 95% CI = 0.65–1.49).

Discussion

In this follow-up study of closely monitored patients with a history of colorectal adenomas, we found that dietary folate intake was inversely related to risk of adenoma recurrence. This association, however, was markedly attenuated after control for dietary fat and fiber intake. Alcohol consumption was a risk factor,

but there was no relationship between cigarette smoking and the risk of recurrence.

Low dietary intake of folate has emerged as a possible risk factor for colorectal cancer or adenomas in four case-control studies (3–6). Two other case-control studies (7,26) have suggested similar effects among women. In one small case-control study among patients undergoing endoscopy (27), levels of folate in red blood cells were lower in adenoma patients than in individuals without adenomas. This finding was replicated among men—but not among women—in a larger case-control study (28). Cohort studies have tended to show less impressive relationships. There were only weak inverse associations between dietary folate intake and adenoma risk among women (7) in the Nurses’ Health Study and no association with colon cancer among men in the Health Professionals’ Study (8). A prospective analysis of colorectal cancer in male smokers (29) reported no association between serum folate levels and colorectal cancer risk.

The relatively little data available regarding folate supplementation do not present a consistent picture. In the Health

Professionals' Study cohort, there were suggestions that folate supplementation was inversely related to risk of colon cancer, whereas dietary folate was not (8). However, multivitamins (which contain substantial amounts of folate) were found to have no effect on adenoma risk by other investigators (30). Total folate intake (including supplements) was inversely related to adenoma risk among women in the Nurses' Health Study cohort (7), but it was not associated with colon cancer risk among men in the Health Professionals' Study cohort (8) and with adenoma risk in a case-control study (28). In male smokers, there were suggestions of an inverse relationship between total folate intake and colon cancer risk and of a direct relationship for rectal cancer (29).

In our data, the association of adenoma risk with dietary intake of folate was substantially attenuated after adjustment for dietary fiber. Similar results were reported in two case-control studies, in which the inverse association of dietary folate with risk of cancer of the colorectum (6) or rectum (5) was attenuated or disappeared after adjustment for intake of other constituents of fruits and vegetables (e.g., vitamin C, vitamin E, and β -carotene). These findings suggest that substances other than folate in fruits and vegetables may explain at least some of their inverse association with the risk of colorectal neoplasia. Our finding that folate-containing foods were not consistently associated with a reduced adenoma risk further supports this conclusion. However, it is possible that low folate intake may have most relevance in combination with other risk factors such as alcohol intake. Also, the effects of folate may also be more pronounced in populations with lower folate intake than in our study population.

Most previous studies have not found an association between cigarette smoking and colorectal cancer [reviewed in (18)]. Some reports have suggested that the risk of colorectal cancer may be increased after a latent period of 20 years or more (19-21,31), a finding that is contradicted by other studies (22,32,33). Previous studies regarding smoking and adenoma risk (13,34-42) are more consistent in finding an increased risk among smokers. Small adenomas seem particularly associated with recent smoking (20,21,43), al-

though a few earlier studies (44-46) reported no association. The reasons for the discrepancies are not clear.

There are experimental data supporting an effect of alcohol consumption on colorectal carcinogenesis (11,12); moreover, epidemiologic evidence has been presented that alcohol intake (especially beer consumption) may be associated with an increased risk of colorectal cancer, particularly rectal cancer (11,12). Most analyses (7,13,39,47) have found alcohol consumption to be a risk factor for colorectal adenomas, although other investigations have reported no association (4,13,38,39,41,48,49) or an association only for large adenomas (37,43). Several studies that considered tumors similar to those that we observed (i.e., small or recurrent adenomas) reported no association with alcohol intake (37,39,43), although this finding was also not universal (7).

Our study has several important strengths. Biased assessment of adenoma occurrence is very unlikely because there was a uniform, blinded pathologic review and all subjects underwent routine endoscopic surveillance, with biopsy of all mucosal lesions. Possible confounding factors, including dietary information, were taken into account. However, our analysis has some limitations. All subjects had a history of at least one adenoma, and it is possible that risk factors for adenoma recurrence differ from those for any adenoma or for colorectal cancer.

Overall, we found inconsistent indications of an anticarcinogenic effect of folate. Although intake of alcohol, one possible folate antagonist, increased the risk of adenoma recurrence, smoking cigarettes, another possible antagonist, did not. Further research, ideally in clinical trials, may be required to clarify the true effect of this nutrient.

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Notes

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