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CD8$^+$ T Lymphocytes are Recruited to Neoplastic Cervix

MARIA C. BELL,[1] ROBERT P. EDWARDS,[1] EDWARD E. PARTRIDGE,[1] KAY KUYKENDALL,[1] WENDY CONNER,[2] HAZEL GORE,[3] ELBA TURBAT-HERRARA,[3] and PEGGY A. CROWLEY-NOWICK[1,4]

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To[1iicIV] distinguish normal cervical lymphocyte populations from phenotypes recruited to the cervix in response to cervical neoplasia, lymphocytes were isolated from normal and neoplastic cervix. A portion of the cervical transformation zone was obtained from 19 patients with pathologically confirmed cervical intraepithelial neoplasia and from 20 patients with normal cervices undergoing hysterectomy for benign indications. Mononuclear cells were harvested from cervical tissue using a serial, multienzymatic digestion procedure and enriched by density gradient centrifugation. Isolated cell populations were stained with surface marker-specific monoclonal antibodies and analyzed by fluorescent activated cell sorter to determine the percentage of B cells, total T cells, CD4$^+$ T cells, CD8$^+$ T cells, and natural killer (NK) cells. The distribution of circulating peripheral blood lymphocyte phenotypes was similar for both patients with neoplasia and normal controls. A marked disparity in the proportions of NK cells and T cells was demonstrated among lymphocyte phenotypes infiltrating the cervix. The percentage of CD4$^+$ T cells and NK cells was significantly depressed (P = 0.04, P = 0.03, respectively) in dysplastic tissue as compared to normal cervical tissue. In contrast, the proportion of CD8$^+$ T cells was significantly increased in the dysplastic tissue (P = 0.0001). Analysis of immunocompetent cells in the circulation appears to have little correlation with immunocytes present in the dysplastic epithelium. The depression in the proportion of CD4$^+$ T lymphocytes and NK cells at the cervical squamocolumnar junction reflects a local recruitment of CD8$^+$ T cells to the site of neoplasia in the cervix.

KEY WORDS: Lymphocyte phenotypes; CIN; transformation zone.

[1] Department of OB/GYN, University of Alabama at Birmingham, 633 20th Street, Birmingham, Alabama 35233.
[2] Department of Biostatistics, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35294.
[3] Department of Surgical Pathology, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35294.
[4] To whom correspondence should be addressed at Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee Womens Research Institute, 204 Craft Avenue, Pittsburgh, Pennsylvania 15213.

INTRODUCTION

Squamous cell carcinoma (SCC) of the cervix represents an ideal system of carcinogenesis to study because well-defined stages of precancerous lesions have been described. In 1983, Durst *et al.* described the first association of HPV type 16 with SCC of the cervix (1). This has since been confirmed by a number of reports (2–5). HPV DNA, particularly of types 16 and 18, is demonstrable in approximately 90% of cervical intraepithelial neoplasia (CIN) lesions and SCC (6). However, as methods of HPV detection have become more sensitive, the HPV-positivity rate is much higher than the rate of clinical disease. The high incidence of HPV infection without clinical disease indicates the presence of HPV DNA is necessary but not sufficient to cause cervical carcinoma (7). The immune response to infection appears to play a central role in the clinical outcome. Epidemiological studies have demonstrated that women with compromised immune systems are more susceptible to progression of HPV infections (8–10), and studies examining regression of HPV infections have demonstrated the importance of cell-mediated immunity (11).

In several tumor models, a more favorable clinical outcome is predicted by the presence of tumor infiltrating lymphocytes (12, 13). Based on the importance of an intact immune response in reverting CIN, the infiltrate of lymphocytes into the site of neoplasia may determine the clinical outcome of infection (persistence, regression, progression). Previous studies of lymphocyte populations infiltrating the dysplastic cervix have used immunohistochemical staining techniques and have demonstrated significant infiltrates in the stroma underlying the lesion (14, 15). However, correlations of disease status and changes in lymphocyte populations have been restricted to studies of peripheral blood lymphocytes (PBL), which have found systemic alterations in cervical carcinoma only (16, 17).

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The lack of availability of dysplastic tissue for isolation of cervical lymphocytes has prevented functional analysis of lymphocytes infiltrating the neoplastic epithelium and subepithelial stroma. However, the recent clinical switch from laser or cryo ablation of CIN lesions to large loop excision of the transformation zone (LLETZ) has led to the availability of dysplastic tissue for study. This type of excision is therapeutic and provides both the dysplastic epithelium and the underlying stromal cells for study. Using an enzymatic digestion procedure, it is possible to isolate lymphocytes that have infiltrated these lesions.

The purpose of this study was to analyze the distribution of lymphocyte phenotypes recruited to the cervix in response to neoplasia compared to normal resident lymphocyte populations found in the cervix. Using a novel approach, viable cervical lymphocytes were isolated, enumerated, and characterized.

MATERIALS AND METHODS

Patients. Cervical tissue and heparinized peripheral blood were obtained from 19 patients with biopsy-proven CIN and 20 patients undergoing hysterectomy for benign gynecologic indications. This study was approved by the Institutional Review Board at the University of Alabama in Birmingham.

CIN patients were scheduled for LLETZ procedure at the University of Alabama, University Hospital colposcopy clinic. Patients ranged in age from 18 to 35 (mean 27.5 ± 6.7). A thorough history including contraception, parity, and smoking history was obtained from each patient at the time of procedure. HIV status was not assessed. All patients enrolled in the study had to meet the following criteria: (1) adequate colposcopy, (2) no suspicion of invasive disease, (3) biopsy-proven CIN, (4) negative endocervical curettage for dysplasia, (5) no previous therapy for CIN, (6) no apparent cervicitis, and (7) nonpregnant at the time of procedure. Based on these criteria, tissue was obtained from 19 women: 6 with CIN I, 11 with CIN II, and 2 with CIN III or CIS.

Hysterectomy patients, normal cervical controls, ranged in age from 26 to 52 (mean 40.2 ± 7.8). Indications for surgery included seven patients with symptomatic fibroids, two patients with pelvic pain, three patients with dysmenorrhea, and eight patients with unspecified indications. These patients had no history of CIN and were negative for dysplasia at the time of surgery based on pathological evaluation.

Isolation of Peripheral Blood Lymphocytes. Peripheral blood mononuclear cells were isolated from heparinized venous blood by density centrifugation on Ficoll-Hypaque. Cells were washed twice with 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). After a final wash in PBS, cells were resuspended at a density of 1×10^6 cells/ml in RPMI 1640 containing 10% fetal calf serum, L-glutamine, penicillin, and streptomycin (complete media).

Isolation of Cervical Lymphocytes. All cervical tissue was rinsed of contaminating residual blood and weighed prior to digestion. Tissue from control subjects was cut to yield tissue representative of the transformation zone, similar to that obtained by LLETZ procedure, and then weighed. Tissue sections were digested using a procedure similar to that described for the isolation of lymphocytes from colon (18). Briefly, the specimens were mechanically disrupted, mincing the tissue into pieces <1 mm square. Four sequential, 30-min cycles of digestion with a multienzymatic digestion media (Joklik's MEM containing 0.05% dispase, 0.002% collagenase, with 4.70 units/ml of hyaluronidase) were performed at 37°C with constant stirring. After each cycle of digestion, the cell suspension was pelleted and washed with PBS to remove enzymes. Tissue debris was removed by a single passage through nylon mesh. The isolated cells were then resuspended in complete media and enriched for mononuclear cells by density centrifugation on Ficoll-Hypaque gradients. Isolated cells were quantitated on a hemacytometer and resuspended in complete media at a concentration of 1×10^6/ml.

Immunochemicals and Staining Procedures. Purified monoclonal antibodies specific for human lymphocyte cell surface antigens (leu 12, specific for CD19, B lymphocytes; leu 4, specific for CD3, T lymphocytes; leu 11c, specific for Fcγ receptor on natural killer (NK) cells; leu 2a, specific for CD8, T suppressor/cytotoxic; and leu 3a, specific for CD4, T helper/inducer) were purchased from Becton Dickinson Laboratories (Burlingame, CA). All antibodies were conjugated with either fluorescein (green) or phycoerythrin (red) and titered to determine the appropriate volume of antibody for maximum fluorescence. Up to a maximum of 1×10^6 cells were pelleted, the supernatant was removed, and the appropriate volume of antibody was added to the pelleted cells. Cells were incubated at 4°C for 30 min, subsequently washed 2× with PBS containing 10% fetal calf serum, and fixed with 1% paraformaldehyde in PBS for analysis. Enumeration of cell populations was performed on a Becton-Dickinson fluorescent activated cell sorter (FACS). The gates were set based on the light scatter characteristics of lymphocytes only, and 10,000 counts were observed.

Statistical Analysis. The data were analyzed using SAS software (SAS/STAT Version 6, Fourth Version).

Duncan's multiple range test was used to compare the ratio of CD4:CD8 between normal and dysplastic groups, and *t* tests were used for all other comparisons of continuous variables. The chi-square test was used for categorical data comparisons. A significance level of 0.05 was used for all comparisons.

RESULTS

All tissue specimens removed by the LLETZ excision procedure were approximately 1.5 × 1.0 cm square with a depth of 0.5–1 cm into the stroma, and a portion of each specimen was obtained for research purposes. Although the tissue specimens were of similar size, the yield of cells varied dramatically between individuals. The yield of cells isolated from patients with CIN ranged from 0.5 × 10^6 to 6 × 10^6. Comparative samples of normal cervix were obtained after surgery from women undergoing hysterectomy. Normal cervical tissue was trimmed to remove excess stroma and resulted in a piece of tissue with a depth similar to the LEETZ specimens. However, a wide variation in cell numbers was also obtained from normal cervical tissue. Although it is possible to isolate mononuclear cells from normal cervical tissue, the quantity of tissue required for isolation of an adequate number of mononuclear cells for analysis was much greater than that from CIN patients. Approximately 50–75% of the normal cervical transformation zone was required for the isolation. The number of mononuclear cells per gram of tissue was significantly greater in the dysplastic epithelium, with a mean of 2.3 × 10^6 ± 0.88 mononuclear cells/g of tissue compared to 0.16 × 10^6 ± 0.8 mononuclear cells/g of tissue in normal cervix (P = 0.04). This difference was not caused by the method used to trim the normal tissue. Replicate samples from four normal cervices were compared to determine the effects of the trimming method (Table I). Although there was some variability between replicates, all cell yields per gram of tissue fell within the range determined for normal cervix on a large group of subjects. This variability did not account for the 10-fold greater yield obtained from CIN tissue. Correlations between grade of CIN and degree of cellular infiltration were not determined due to the limited number of patients in each group.

Table I. Comparison of Mononuclear Cells Yields from Replicate Samples of Normal Cervical Tissue[a]

Patient	Tissue weight (g)	Mononuclear cells/g
1	1.59	0.125 × 10^6
	2.92	0.147 × 10^6
2	2.42	0.157 × 10^6
	1.55	0.348 × 10^6
3	0.67	0.59 × 10^6
	2.02	0.09 × 10^6
4	1.01	0.257 × 10^6
	1.26	0.094 × 10^6

[a] Mean cell yield from: 20 normal subjects 0.16 × 10^6 ± 0.8 cells/g; 18 CIN patients 2.3 × 10^6 ± 0.88 cells/g.

The viability of cells from both CIN patients and normal controls was 90–98% at the completion of the isolation procedure as determined by trypan blue exclusion. A comparison of the size and granularity of the cells by FACS scan analysis after isolation and enrichment demonstrated that the majority of cells were lymphocytes (Fig. 1). All further analysis was performed on this population of cells.

Comparison of B cells, T cells, and NK cells isolated from CIN tissue and normal cervix revealed dramatic differences (Fig. 2). The percentage of infiltrating T cells was significantly increased in dysplastic epithelium compared to benign cervical tissue (P = 0.0001). The proportion of NK cells was significantly depressed in the dysplastic cervical tissue from CIN patients compared to normal cervical tissue (P = 0.03). There was no demonstrable difference in the percentage of B cells infiltrating dysplastic or normal cervical tissue.

Analysis of T-cell subpopulations, $CD4^+$ and $CD8^+$ cells, also demonstrated significant differences between dysplastic infiltrating lymphocytes and normal resident lymphocytes. $CD4^+$ T cells in dysplastic epithelium and subepithelial stroma had a lower mean percentage at 1.72 ± 0.34% compared to normal tissue, which was 3.76 ± 0.09% (P = 0.04). Conversely, $CD8^+$ T cells in dysplastic tissue had a higher percentage with a mean of 17.7 ± 1.72% compared to 4.68 ± 0.45% in normal tissue (P = 0.0001; Fig. 3). The CD4:CD8 ratio was significantly depressed (P = 0.009) in the CIN lesions. This difference was not detected when the ratio from PBL was analyzed between the two groups.

To test for systemic phenotype alterations and to examine correlations between PBL populations and cervical infiltrating lymphocytes, matched PBL samples were analyzed from each patient. Comparisons of the percentage of B cells, T cells, $CD8^+$ T cells (Ts/c), $CD4^+$ T cells (Th/i), and NK cells in peripheral blood demonstrated no statistically significant differences between CIN patients and normal controls (data not shown). In addition, no correlation between PBL phenotypes and cervical lymphocyte phenotypes was observed for either the normal or dysplastic cervical tissue.

A proportion of the $CD3^+$ cells isolated from both normal and neoplastic cervix did not express CD4 or CD8. To confirm this observation and that the difference

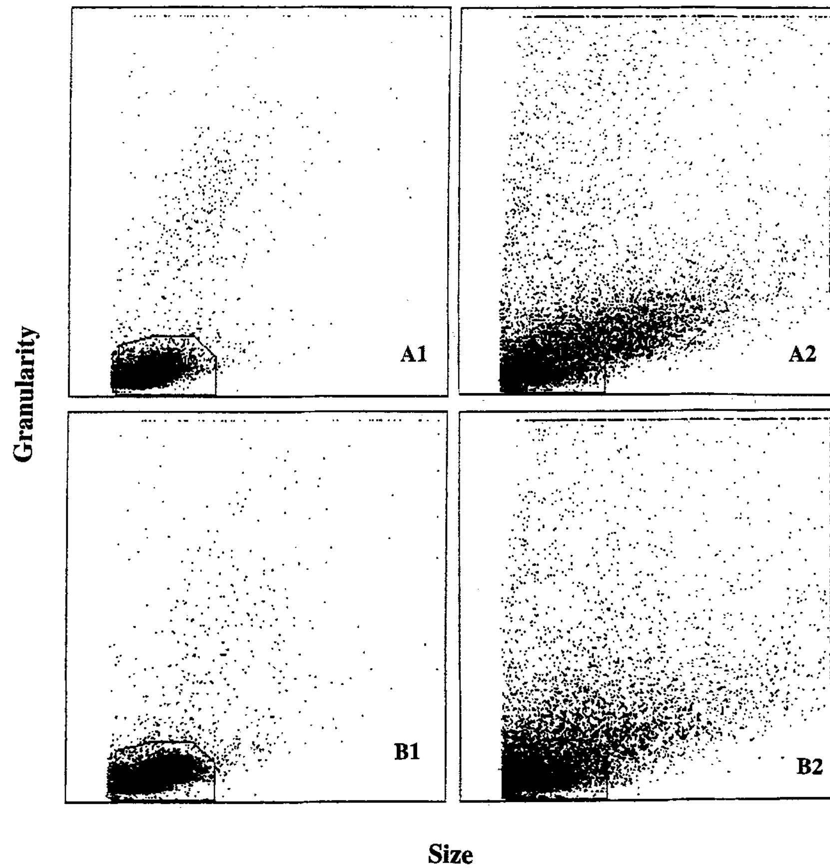

Fig. 1. Two representative FACS scans depicting the size and granularity of cells isolated from peripheral blood and cervix. A1 and A2 demonstrate the light scatter of cells isolated from peripheral blood and cervix, respectively from a patient undergoing hysterectomy for benign indications, and B1 and B2 are from a patient with CIN II and represent mononuclear cells from peripheral blood and cervix, respectively. The boxes enclose cells which have the light scatter characteristics of lymphocytes; these cells were used for the analysis.

in the tissue lymphocytes and PBL was not an artifact of the digestion procedure, several controls were performed. The effect of the digestion process on lymphocyte surface antigens was examined by subjecting isolated PBLs to the digestion media for one round (30 min) of digestion and then staining with the panel of monoclonal antibodies. The distribution of lymphocyte populations was unchanged by the procedure. To control for cleavage of surface antigens by digestion enzymes, cervical tissue was digested, mononuclear cells were isolated, and cell isolates were divided into two cultures. Half the cells were stained immediately for CD3, CD4, and CD8 markers; the remaining cells were incubated 4 hr at 37°C to allow for the regeneration of specific surface markers that may have been cleaved. This control demonstrated no changes in the percentages of T cells or T-cell subsets analyzed after regeneration of surface receptors. Lymphocyte phenotypes were also determined for PBL before and after mesh filtration to assess the effect on cellular populations. No change was noted in the phenotype distribution.

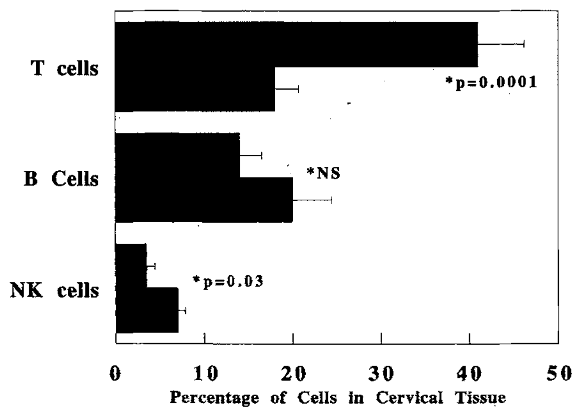

Fig. 2. The mean percentage of NK cells, T lymphocytes, and B lymphocytes infiltrating cervical epithelium of patients with CIN and normal cervical tissue. Black bar represents CIN patients and gray bar represents normal subjects.

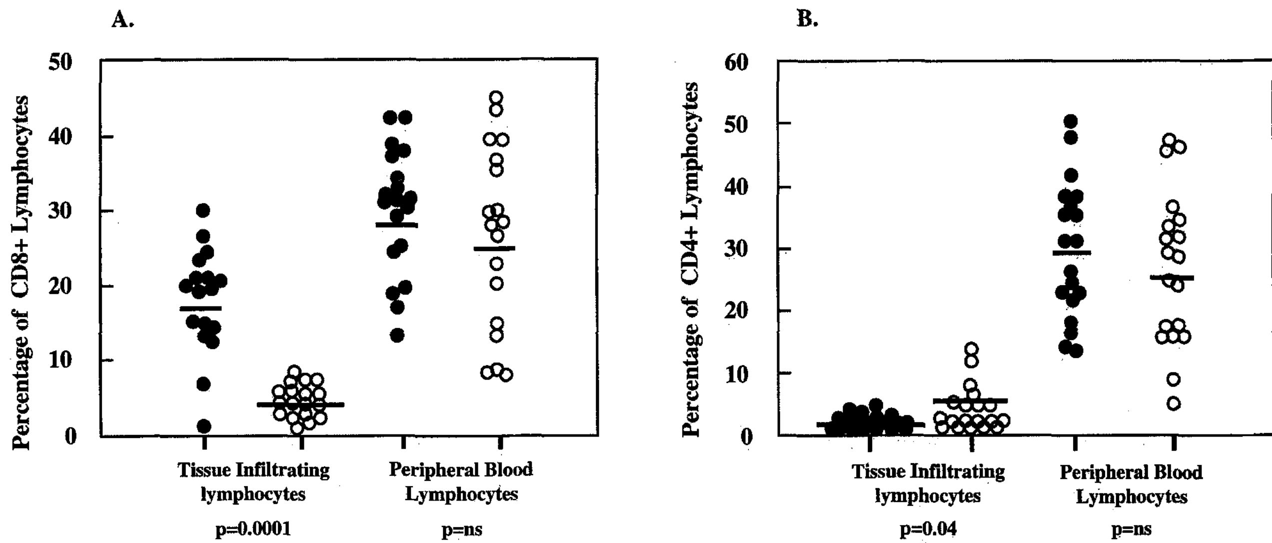

Fig. 3. The percentage of $CD8^+$ (A) and $CD4^+$ (B) cells isolated from cervix and peripheral blood of individual patients with CIN (closed circles) or normal cervix (open circles) are depicted.

DISCUSSION

Depression of immune function has been demonstrated to be a risk factor for progression of cervical neoplasia. Previous studies have described altered populations of circulating lymphocytes in patients with cervical carcinoma (16, 19). However, the circulating response in women with CIN has not been described, and the relationship of circulating lymphocyte populations to lymphocytes infiltrating the site of infection the neoplastic lesion has not been examined. In this study, lymphocyte isolates from dysplastic cervical epithelium and subepithelial stroma were subjected to surface marker analysis. These findings were compared to those of normal cervical lymphocytes as well as circulating PBL. The rationale for such an approach was to differentiate the local phenomena from systemic phenomena. If neoplasia is associated with an immunomodulation, a local alteration in lymphocyte phenotypes would be expected as preinvasive lesions progress. These alterations should be demonstratable long before a lesion-induced systemic effect is observed, as previously demonstrated in patients with invasive carcinoma.

Analysis of circulating lymphocyte phenotypes demonstrated no differences between women with CIN and healthy individuals, indicating that systemic changes may only occur in advanced stages of carcinoma. In addition, no correlation between circulating lymphocyte populations and local tissue infiltrating lymphocyte phenotypes in women with CIN was found. Yet, dramatic differences in both the number and phenotype of lymphocytes isolated from normal cervical and CIN tissue were revealed.

Normal cervical epithelium and stroma are populated by predominantly B cells and a smaller proportion of T and NK cells. The data presented in this report indicate that recruitment of lymphocytes occurs as neoplastic changes develop in the cervix presumably due to the expression of HPV antigens and specific cytokines. $CD8^+$ T cells and B cells infiltrate, resulting in a decrease in the proportion of NK cells and $CD4^+$ T cells present locally in the cervix. This decrease is most likely caused by the influx of $CD8^+$ T cells and not a change in the absolute number of NK cells or $CD4^+$ T cells. This finding is similar to results of several reports describing the infiltration of lymphocytes into dysplastic cervix using immunohistochemical techniques to analyze cervical infiltrates. In these studies tissue from women with abnormal Pap smears (presumably early HPV infection) who had normal biopsies were compared to those with neoplasia in their biopsy (advanced HPV infection). These immunohistochemical studies demonstrated similar lymphocyte populations in early and advanced CIN lesions, indicating that recruitment of lymphocyte populations may occur immediately upon infection with HPV even in low grade lesions (14, 15). Our data on isolated lymphocytes from both normal and HPV infected cervix support and strengthen this conclusion.

The recruitment of T lymphocyte subpopulations, specifically $CD8^+$ T cells, into the cervix may play a key

role in the outcome of these lesions. In a normal cervix the ratio of CD4:CD8 cells is approximately 1.0 in the stroma and slightly lower in the epithelium (14, 20). The ratio of CD4:CD8 infiltrating lymphocytes isolated from CIN patients in our study was significantly depressed compared to normal tissue, which represents an infiltration of $CD8^+$ T cells. A similar depression in the CD4:CD8 ratio has been described for cervical carcinoma, carcinoma *in situ*, and patients with biopsy-proven CIN by immunohistochemical analysis (14, 15, 21).

Infiltration of $CD8^+$ T cells into the dysplastic cervix correlates well with reports of cervical carcinoma and other forms of carcinoma such as ovarian, colorectal, and renal cell (22–25). It is interesting that an infiltration of $CD8^+$ cells could be detected in early stages of dysplasia. If HPV antigens are presented properly on the surface of infected epithelial cells, it would be expected that these infiltrating $CD8^+$ cells would have some cytolytic activity. However, it has been demonstrated in a number of human and experimental systems that tumor infiltrating lymphocytes, which are predominantly $CD8^+$ T cells, have impaired effector functions (26, 27). The ability to isolate CIN infiltrating lymphocytes will allow investigators to analyze the functional capabilities of these cells and determine if T-cell anergy occurs during early stages of neoplasia.

An unexpected observation was the detection of a large proportion of $CD3^+$ cells, which were CD4 and CD8 negative (20–35%). This phenomenon has been described for other mucosal sites but with a smaller percentage of cells (28). At alternative sites these cells have been shown to express $\gamma\delta$ T-cell receptors. The $CD3^+$ cells in normal and neoplastic cervix do not utilize this T-cell receptor. Further analysis of this cellular population is currently underway.

Unlike T lymphocytes, which require specific antigen priming and sensitization by accessory cells to become activated, NK cells have a spontaneous cytotoxic effect against virally infected cells and neoplastic cells (29). In normal cervical tissue, NK cells are distributed throughout the basal epithelium, and stroma. In CIN lesions, the distribution is more diffuse; NK cells are found throughout the epithelium (30). An increase in NK cell infiltration into the stroma in patients with dyskaryosis (31) and CIN (30) has been described. Our data demonstrated a decrease in NK percentage in CIN tissue compared to normal cervix, which may be related to the influx of T lymphocytes, not to a decrease in absolute cell number.

The data presented in this report demonstrate that local immune infiltrates are dramatically different in CIN lesions compared to normal cervix, which emphasizes the need to evaluate local infiltrating lymphocytes within the lesion to understand the immune response to HPV infection and cervical neoplasia. A number of T-cell epitopes have been identified on HPV16 E6 and E7 proteins. It is possible that a percentage of these infiltrating lymphocytes are specific for these antigens. The isolation of lymphocytes from the transformation zone of women with cervical neoplasia and healthy cervix represents the first analysis of isolated cervical lymphocyte populations. This technique will provide investigators with a reliable new approach to examine viable infiltrating lymphocytes. Further analysis of these cells from CIN patients should provide information about the effector function and immune specificity of infiltrating lymphocytes.

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