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Phenotypical Characterization of Lymphocytes Infiltrating Regressing Papillomas

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Papillomavirus-induced lesions often regress spontaneously in both humans and animals. Papilloma regression is deemed to be due to a cell-mediated immune response, the nature of which is still ill defined, and is accompanied by immune cell infiltrates. To gain further information on the nature and role of the immune cells present in regressing papillomas, we have analyzed biopsies of papillomas induced in the soft palate of cattle by bovine papillomavirus type 4 (BPV-4) and have phenotypically characterized and quantified the lymphocytes present in these lesions. Eleven papilloma biopsies and seven biopsies of noninfected palate were analyzed for the presence of activated $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ lymphocytes. We found large numbers of lymphocytes in the subepithelial derma of papillomas but not in normal palate tissue; these cellular masses consisted predominantly of $CD4^+$ lymphocytes, with only a few $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes, generally positioned at the periphery of these masses. All three subtypes of lymphocytes were found interdigitated with the cells of the basal layer both in papillomas and in normal palate tissue, but while basal layer $CD8^+$ and $\gamma\delta(WC1^+)$ T cells were detected with similar frequencies in papillomas and uninfected palate, basal layer $CD4^+$ T cells were much more frequent in papillomas. $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ lymphocytes were found in the suprabasal layers of papillomas, but the $CD8^+$ and $\gamma\delta(WC1^+)$ T cells were more numerous and had migrated further into the differentiating keratinocytes of the papilloma fronds than the $CD4^+$ T cells. We conclude that T-cell infiltration is characteristic of regressing BPV-4 papillomas, that $CD4^+$ lymphocytes are specifically and massively recruited into the regressing papillomas, and that although all three lymphocyte subsets can penetrate the papilloma, only the $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes are able to migrate into the fronds. These results suggest that all three lymphocyte subsets have an important role to fulfill during natural regression of papillomas.

Papillomaviruses infect mucous and cutaneous epithelia and induce hyperplastic lesions, defined as warts, papillomas, or condylomas. These lesions are generally benign, but if they are accompanied by other cellular changes, often induced by exposure to environmental carcinogens, then cancers can develop (13). Natural regression of papillomas has been found in humans and animals (15, 19, 24, 27), and histological examination of the regressing lesions has revealed marked infiltration of lymphocytes in both the epithelium and the dermis. Experimentally regression of papillomas can be induced in animals by immunization with defined viral antigens (2, 16, 26), and also in these cases, regression is accompanied by infiltrates of immune cells. These results, coupled with the observation that papillomas and other papillomavirus-induced lesions do not regress in individuals whose cellular immune responses are compromised (1), strongly suggest that cellular immunity plays an important role in their regression. However the precise immunological basis for the rejection of papillomas is still poorly understood.

Bovine papillomavirus type 4 (BPV-4) infects the mucous epithelium of upper alimentary canal of cattle and induces benign papillomas which in immunocompetent animals are rejected approximately 1 year after the onset of infection (15). In cattle chronically immunosuppressed by a diet of bracken fern, the papillomas spread and persist and become a focus for neoplastic progression to squamous cell carcinomas (3), confirming that the integrity of the immune system is pivotal to the ability of the host to undergo tumor rejection. Experimental rejection of BPV-4 papillomas has been achieved by immunizing cattle with the viral protein E7 (2), and the regressing papillomas showed infiltrates of immune cells similar to those observed in naturally regressing tumors.

The BPV-4 system is the most extensively studied animal model of mucosal papillomavirus infection applicable to the anogenital and respiratory epithelia in humans. As the immune mechanisms that control BPV-4 infection in cattle are likely to be similar to those in humans, an investigation of the events occurring during BPV-4 papilloma rejection should shed light on the rejection processes occurring in humans. To this end, we have analyzed the lymphocyte populations infiltrating naturally regressing BPV-4 papillomas, and here we report on their phenotype and distribution within the lesions.

MATERIALS AND METHODS

Animals and infection with BPV-4. Young calves (8 to 12 weeks old) were infected in the soft palate by intradermal inoculation of 10^{11} particles of BPV-4 in 10 sites (10^{10} particles per site) (2). The mouths were examined for lesions at intervals of 4 to 6 weeks. After approximately 32 weeks, papillomas that were deemed to be undergoing rejection by macroscopic criteria were biopsied with the underlying derma. Biopsies from the soft palate of control noninfected animals were also taken.

Histology. Biopsies were bisected; one half were snap-frozen by immersion in liquid nitrogen for immunocytochemical analysis, the other half were fixed in buffered formalin for subsequent embedding in paraffin wax. Paraffin blocks were sectioned and then stained with hematoxylin and eosin.

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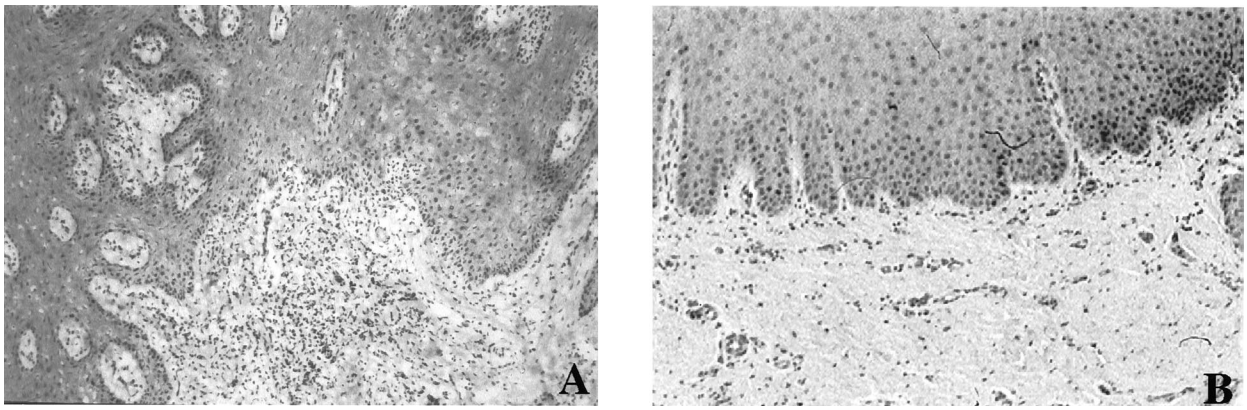

FIG. 1. Hematoxylin-and-eosin staining of histological sections of regressing papilloma 78/P2 (A) and a nonregressing papilloma (B). Magnification is ×40.

Immunocytochemistry. Cryostat sections were cut from the snap-frozen papillomas, air dried, and then stored at −20°C. Sections were thawed and fixed in chilled acetone before being stained with the following monoclonal antibodies (MAbs): CC30 (anti-CD4), CC63 (anti-CD8) (12), CC15 (anti-WC1 antigen of γδ T cells [5]), and ILA-111 (anti-α-chain interleukin-2 receptor [IL2-R] [22]). Prior to use on cryosections, all of these MAbs were shown by fluorescence-activated cell sorting to be reactive against bovine peripheral blood lymphocytes (not shown). MAbs were incubated with sections for 1 h at room temperature in a moist chamber. Sections were then washed three times with phosphate-buffered saline, and bound antibodies were revealed by using peroxidase-conjugated reagents from a Vector ABC Elite kit specific for mouse antibodies, with diaminobenzidine as the chromogen. The tissue sections counterstained with eosin.

Hematoxylin-and-eosin staining of two sections cut at the beginning and end of each run of frozen sections was used for measuring area and length of epithelium.

Enumeration of cell types. The sections of papillomas and uninfected palate were divided into four regions: region 1, the subepithelial derma; region 2, the basal cell layer; region 3, the invaginations of the derma into the body of the papilloma (this region is contiguous with the subbasal region defined as region 1); and region 4, the area above the basal cell layer (excluding region 3), primarily comprising differentiating keratinocytes. In many sections, fixed boundaries of region 1 were often difficult to define, and the lymphocytes were too numerous to count; therefore numerical data from this region were not generated. In serial sections, regions 2, 3, and 4 formed relatively constant areas in which to count $CD4^+$, $CD8^+$, $\gamma\delta(WC1^+)$, and IL2-R^+ lymphocytes, and therefore quantitative data are provided for these regions. For each biopsy specimen, the areas of regions 3 and 4 and the length of the basal cell layer were calculated from two sections that were stained with hematoxylin and eosin. The microscope slides were screened in a slide scanner; the image was manipulated by using Adobe Photoshop and analyzed by using an NIH package. A microscope slide with a 1-mm line etched on it was used to calibrate the images. For each specimen, the mean of the two slides was taken. The densities for each type of lymphocyte are expressed as cell numbers per square millimeter in regions 3 and 4 and as cell numbers per millimeter in region 2.

Statistical analysis of lymphocyte counts. Differences in the density of $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ lymphocytes among regions 2, 3, and 4 were analyzed by using paired *t* tests. Paired *t* tests are appropriate because the comparisons are based on individual differences in cell counts between two regions within the same specimen. The paired differences were then combined for the papillomas or uninfected palatine biopsies. Differences in density of lymphocyte subtypes in a particular region between the papillomas and the uninfected palate samples were analyzed by using an unpaired *t* test.

RESULTS

Eleven papillomas and seven control palate samples were stained with the panel of MAbs; only photographs from representative papillomas are presented.

Histological analysis. Histological examination of the papilloma biopsies clearly demonstrated mononuclear infiltrates in all of them. In some biopsies, the infiltration was particularly severe, with large masses of lymphocytes in the subbasal derma (Fig. 1A). In contrast, no infiltrate was observed in nonregressing papillomas (Fig. 1B) or in noninfected palate (see Fig. 5).

Distribution of lymphocyte subtypes in regressing papillomas. In regressing papillomas, the $CD4^+$ lymphocytes were predominantly distributed in regions 1 (cell counts not given) and 3, that is, in the derma and its invaginations. Few $CD4^+$ lymphocytes were present in the basal layer (region 2), interdigitated with the epithelial cells, and were only sporadically found in region 4 (Fig. 2B and Table 1). $CD8^+$ lymphocytes were also found in regions 1, 2, and 3 and in relatively large numbers also in region 4, among the keratinocytes of the suprabasal layers (Fig. 2C and F and Table 1). The pattern of $\gamma\delta(WC1^+)$ lymphocytes distribution was similar to that of $CD8^+$ lymphocytes (Fig. 2D and Table 1).

In contrast to the epithelial cells of the basal layers of the papilloma, the great majority of lymphocytes was negative for Ki-67, a MAb specific for a nuclear antigen associated with cell proliferation (8) (Fig. 2E), indicating that the lymphocytes were not proliferating in situ.

The mean densities of each type of lymphocyte in regions 2, 3, and 4 are shown in Table 1. Within region 1, there was a larger population of $CD4^+$ than of $CD8^+$ cells; although these cells were too numerous to count, this conclusion is confirmed by the greater density of $CD4^+$ than of $CD8^+$ cells in region 3, which is contiguous with region 1. There were no significant differences in the numbers of $CD4^+$ and $CD8^+$ cells within the basal cell layer (region 2). In region 4, there were significantly higher densities of $CD8^+$ than of $CD4^+$ cells. The densities of $\gamma\delta(WC1^+)$ lymphocytes in regions 2, 3, and 4 were generally higher than those of $CD8^+$ lymphocytes. The *P* values for these differences are shown in Table 2.

In some papillomas, there were large foci of lymphocytes situated either just beneath or straddling the basal cell layer. Although within each focus the densities of cells were too great to allow precise counts, it was obvious that the overwhelming majority of cells consisted of $CD4^+$ lymphocytes (Fig. 3A). $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes were also present, although they were not evenly distributed within the cluster of $CD4^+$ cells but restricted to the fringes of each focus (Fig. 3B and C). In two cases, as illustrated by papilloma 331/P1 in Fig. 4, these clusters of lymphocytes appeared to destroy the integrity of the basal cell layer and to infiltrate deep in the papilloma.

Distribution of lymphocyte subtypes in uninfected palate. The distribution and mean densities of lymphocytes in uninfected palatine biopsies are shown in Fig. 5 and Table 1. There were no appreciable lymphocyte deposits in the subepithelial derma. There were significantly higher densities of $CD8^+$ than of $CD4^+$ cells in regions 2 and 4, and the $\gamma\delta(WC1^+)$ lymphocytes were more numerous than $CD4^+$ cells in all regions and more numerous than $CD8^+$ cells in regions 2 and 3. The *P* values for the observed differences are shown in Table 2.

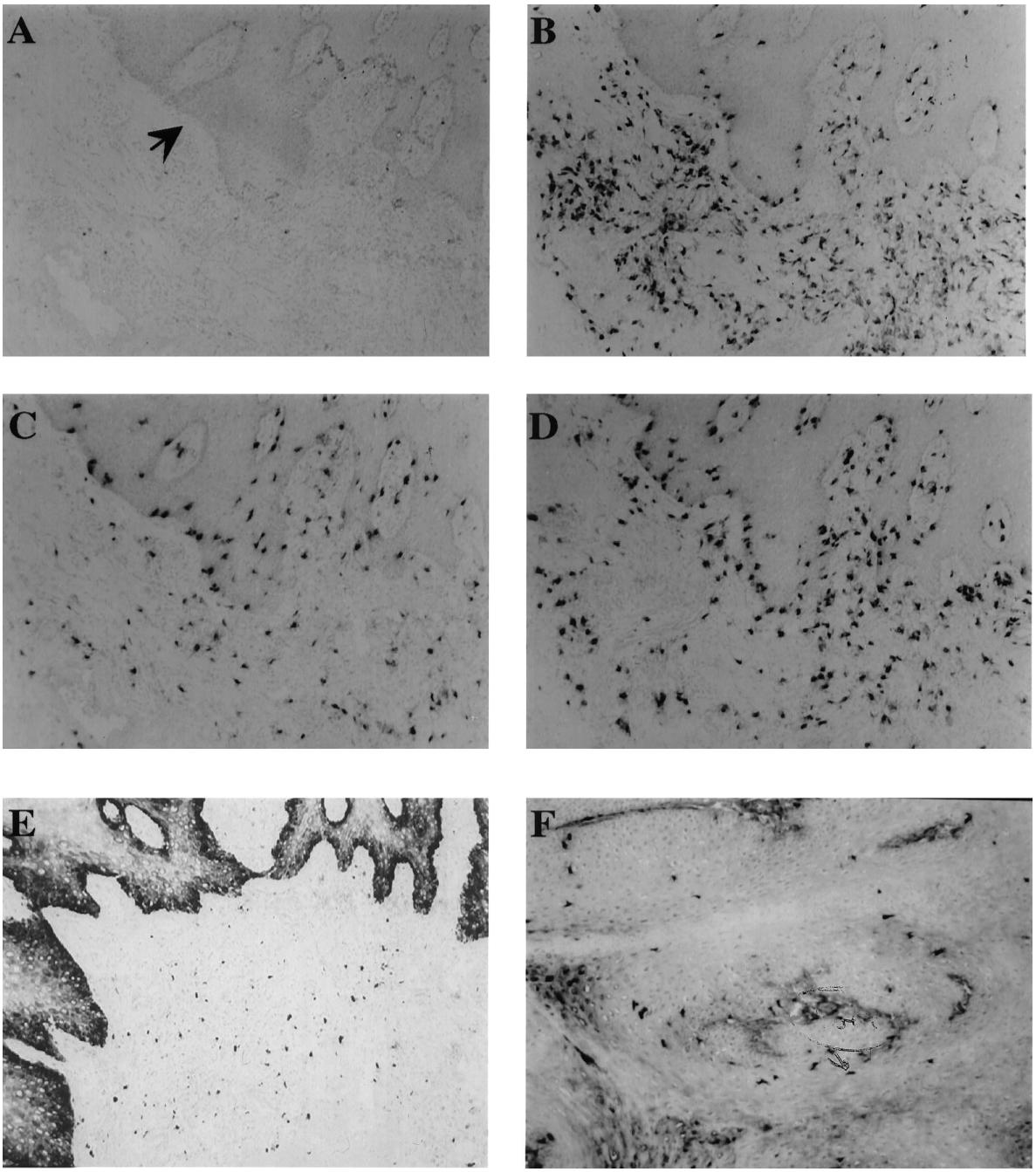

FIG. 2. Lymphocyte subtypes in papillomas. (A to D) Serial histological sections of papilloma 78/P2 stained with no primary antibody (A), anti-CD4 MAb CC30 (B), anti-CD8 MAb CC63 (C), and anti-WC1(γδ) MAb CC15 (D). (E) Histological section of papilloma 78/P2, serial to that shown in Fig. 1A, stained with MAb Ki-67. (F) Histological section of papilloma 358/P5 stained with anti-CD8 MAb CC63. Magnifications are ×50 in panel E and ×100 in the other panels. The arrow in panel A points to the basal layer.

Differences in densities of lymphocytes between regressing papillomas and uninfected palate. A comparison of the mean densities of lymphocytes between regressing papillomas and uninfected palatine biopsies is shown with *P* values in Table 1. The major differences in lymphocyte densities were found in region 1, where no large deposits were seen in normal palatine biopsies, and in region 4, where the densities of $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ lymphocytes were significantly higher in papillomas than in the uninfected palates. In regions 2 and 3, only the densities of $CD4^+$ cells in papillomas exceeded that of uninfected palates, and there were no significant differences in the number of $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes in these regions.

$CD4^+$, $CD8^+$, and $\gamma\delta^+$ lymphocytes express IL2-R. Activated lymphocytes express the α and β chains of IL2-R (28). To establish the state of activation of the lymphocyte subtypes in papillomas, sections were stained with MAb ILA-111, which specifically recognizes the α chain of IL2-R. Double-staining

TABLE 1. Density differences of lymphocyte subtypes between papillomas and uninfected palates

Population	Papillomavirus region[a]	Mean density ± SEM in:		*P* value[b]
		Papillomas	Palates	
$CD4^+$	2	2.4 ± 0.6	0.4 ± 0.05	**0.012**
	3	20 ± 5.5	3 ± 0.7	**0.016**
	4	4.5 ± 1.43	0.1 ± 0.06	**0.016**
$CD8^+$	2	2.4 ± 0.4	2.7 ± 0.6	0.78
	3	12.8 ± 3.8	6.5 ± 1.2	0.17
	4	17.2 ± 5.4	3 ± 0.6	**0.036**
$\gamma\delta(WC1^+)$	2	6.7 ± 1.3	5.7 ± 0.7	0.56
	3	22.6 ± 4.7	14.5 ± 1.6	0.16
	4	15.1 ± 3.2	4.7 ± 0.5	**0.013**
IL2-R	2	5 ± 1.4	4.2 ± 0.8	0.67
	3	23.2 ± 7	0	
	4	17.3 ± 5.7	0	

[a] Described in Materials and Methods.
[b] Significant *P* values are in boldface.

with ILA-111 and either CC30, CC63, or CC15 was unsuccessful; therefore, staining of serial sections had to be used. First, the ability to detect individual types of lymphocytes on sequential sections was investigated. For one specimen, three sequential sections were stained for $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ lymphocytes (nine slides in all). Sections were photographed, and the positions of a minimum of 25 individual lymphocytes (often more if heavily infiltrated) in the first section were noted; these positions were then checked in the second and third sections. The percentages detected for $CD4^+$, $CD8^+$, and $\gamma\delta(WC1^+)$ were 75, 60, and 82, respectively, in the second section and 25, 24, and 26, respectively, in the third section. Having verified that most lymphocytes could be identified in pairs of serial sections, these sections were stained with ILA-111 and either CC30 ($IL2\text{-}R^+$ and $CD4^+$), CC63 ($IL2\text{-}R^+$ and $CD8^+$), or CC15 [$IL2\text{-}R^+$ and $\gamma\delta(WC1^+)$]. Many lymphocytes in all regions of the papillomas, including the large foci in the subepithelial derma, expressed IL2-R (Fig. 3D and 4D and Table 1). $IL2\text{-}R^+$ lymphocytes were counted in regions 2 and 4. Forty percent of $CD4^+$, 33% of $CD8^+$, and 62% of $\gamma\delta(WC1^+)$ lymphocytes were $IL2\text{-}R^+$, indicating that, allowing for the percentage of cells detected in the second section, approximately half of $CD4^+$ and $CD8^+$ lymphocytes and three-quarters of $\gamma\delta(WC1^+)$ lymphocytes expressed IL2-R and were therefore activated. In one papilloma, the lymphocytes appeared to be $IL2\text{-}R^-$; the significance of this is not understood.

In contrast to papillomas in which $IL2\text{-}R^+$ lymphocytes were found in all areas, in normal palatine biopsies only lymphocytes in region 2 were $IL2\text{-}R^+$ (Table 1).

TABLE 2. Density differences between lymphocyte subtypes in different epithelial regions

Lymphocyte subtype	Papillomavirus region[a]	Mean difference between pairs[b]	*P* value[c]
Papillomas			
$CD8^+$ vs $CD4^+$	2	0.05	0.93
	3	−7.25	**0.013**
	4	12.64	**0.032**
$\gamma\delta^+$ vs $CD4^+$	2	4.3	**0.0006**
	3	2.54	0.14
	4	10.57	**0.0006**
$\gamma\delta^+$ vs $CD8^+$	2	4.25	**0.0039**
	3	9.78	**0.0012**
	4	−2.07	0.74
Palate biopsies			
$CD8^+$ vs $CD4^+$	2	2.23	**0.037**
	3	3.55	0.093
	4	2.84	**0.014**
$\gamma\delta^+$ vs $CD4^+$	2	5.27	**0.0016**
	3	11.53	**0.0005**
	4	4.55	**0.0012**
$\gamma\delta^+$ vs $CD8^+$	2	3.04	**0.031**
	3	8.0	**0.027**
	4	1.71	0.073

[a] Described in Materials and Methods.
[b] Calculated as the sum of (density of cell type a) − (density of cell type b)/number of pairs. A negative value indicates that the density of cell type a is smaller than the density of cell type b.
[c] Significant *P* values are in boldface.

DISCUSSION

Natural regression of papillomavirus-induced tumors is accompanied by marked infiltration of lymphocytes (6, 15, 23, 24, 27). Similar infiltrates have been observed in papillomas regressing as a result of either vaccination (16) or intralesion immunotherapy with *Corynebacterium parvum* (9). The precise mechanism of rejection is not known, nor is it known whether the lymphocytes are activated against viral or cellular antigens, although in the case of vaccine-induced regression, lymphocytes proliferate in vitro in response to the immunizing viral antigen (20).

As part of our investigations into the mechanisms of papilloma rejection in the BPV-4 system, we have analyzed the phenotype of the T lymphocytes infiltrating naturally regressing papillomas. We have repeatedly observed that natural regression of upper alimentary canal papillomas starts approximately 8 to 12 months after infection. Indeed, papillomas that were deemed to have entered rejection by visual inspection were shown by histopathological analysis to be massively infiltrated by immune cells. This contrasts with the absence of such heavy infiltrates in nonregressing papillomas and in uninfected palate, where many fewer lymphocytes can be detected. The presence of large numbers of lymphocytes does seem therefore to correlate with the regressing status of the lesions. A similar conclusion has been reached for regressing papillomas induced by cottontail rabbit papillomavirus (23) and for regressing genital condylomas in humans (6). In both cases, regressing lesions were infiltrated by lymphocytes, which were much more numerous than in noninfected epithelium or nonregressing lesions. As in regressing cottontail rabbit papillomavirus warts (23), the lymphocytes in regressing BPV-4 papillomas were not proliferating, suggesting that their activation and expansion take place elsewhere.

In regressing BPV-4 papillomas, there were discernible differences in the types of lymphocytes that populated different regions of the lesions. The predominant subtype of T lymphocyte in these papillomas was by far the $CD4^+$ cell. The great majority of $CD4^+$ cells were present in the subepithelial derma, where they often formed clusters. Some $CD4^+$ cells were also located interdigitating with the cells of the basal and suprabasal layers, but in these regions, $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes were more prominent. In one papilloma, the $CD8^+$ lymphocytes were found among the highly differentiated frond keratinocytes, adjacent to the keratin layer. This differential distribution would suggest that either $CD4^+$ lymphocytes are less able than other lymphocyte subtypes to migrate across the basal cell layer and into the body of the papilloma or that $CD8^+$ and $\gamma\delta(WC1^+)$ lymphocytes are specifically attracted into the suprabasal layers. Since $CD8^+$ lymphocytes are generally regarded as the major cytolytic cells, their proximity

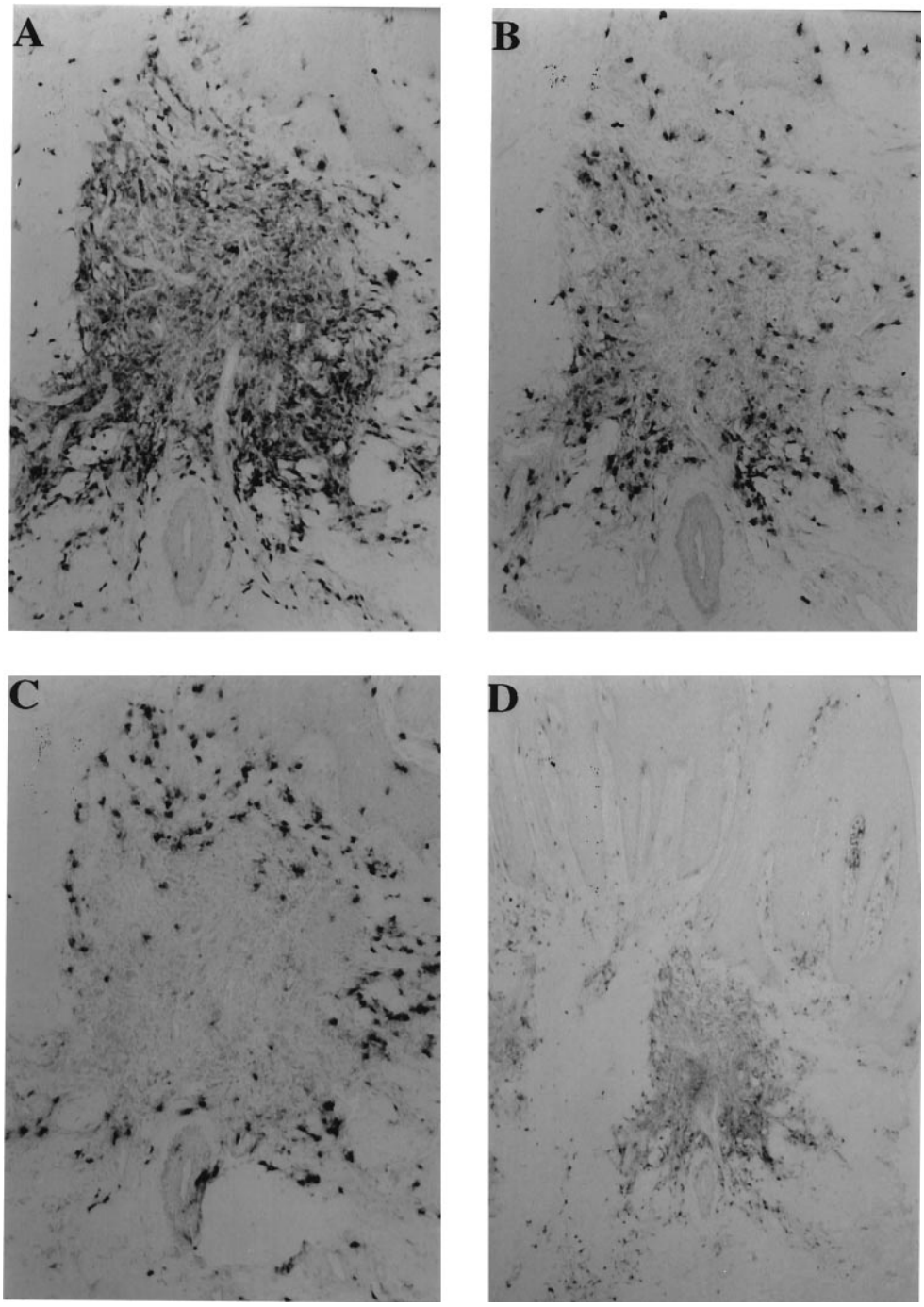

FIG. 3. Lymphocyte subtypes in a cell cluster in subbasal space of papilloma 78/P2. Shown are serial histological sections stained with anti-CD4 MAb CC30 (A), anti-CD8 MAb CC63 (B), anti-WC1(γδ) MAb CC15 (C), and anti-IL2-R MAb ILA-111 (D). Magnifications are ×100 in panels A to C and ×40 in panel D. Both derma and papilloma are noticeable in panel D.

to infected keratinocytes would potentially allow the killing of infected cells. Lymphocytes were also found among the differentiated keratinocytes in regressing cottontail rabbit papillomavirus papillomas, but their phenotype was not established (23). In regressing genital condylomas in humans, large numbers of $CD4^+$ and $CD8^+$ cells are found within both the stroma and the surface epithelium of the lesion, but γδ lymphocytes were not investigated (6). The preponderance of $CD4^+$ cells lead to the suggestion that regression of genital warts was mediated by $CD4^+$ lymphocytes and was probably due to a delayed-type hypersensitivity response (6), in analogy to what was previously observed in experimental systems (4, 21) and in rabbits with regressing papillomas (11). Also in regressing BPV-4 papillomas, the massive recruitment of $CD4^+$ lymphocytes in the subbasal derma, and occasionally intraepithelially, points to an important involvement of these cells in regression.

Ruminants have large amounts of γδ lymphocytes both as newborns and as adults (10). These cells are particularly prom-

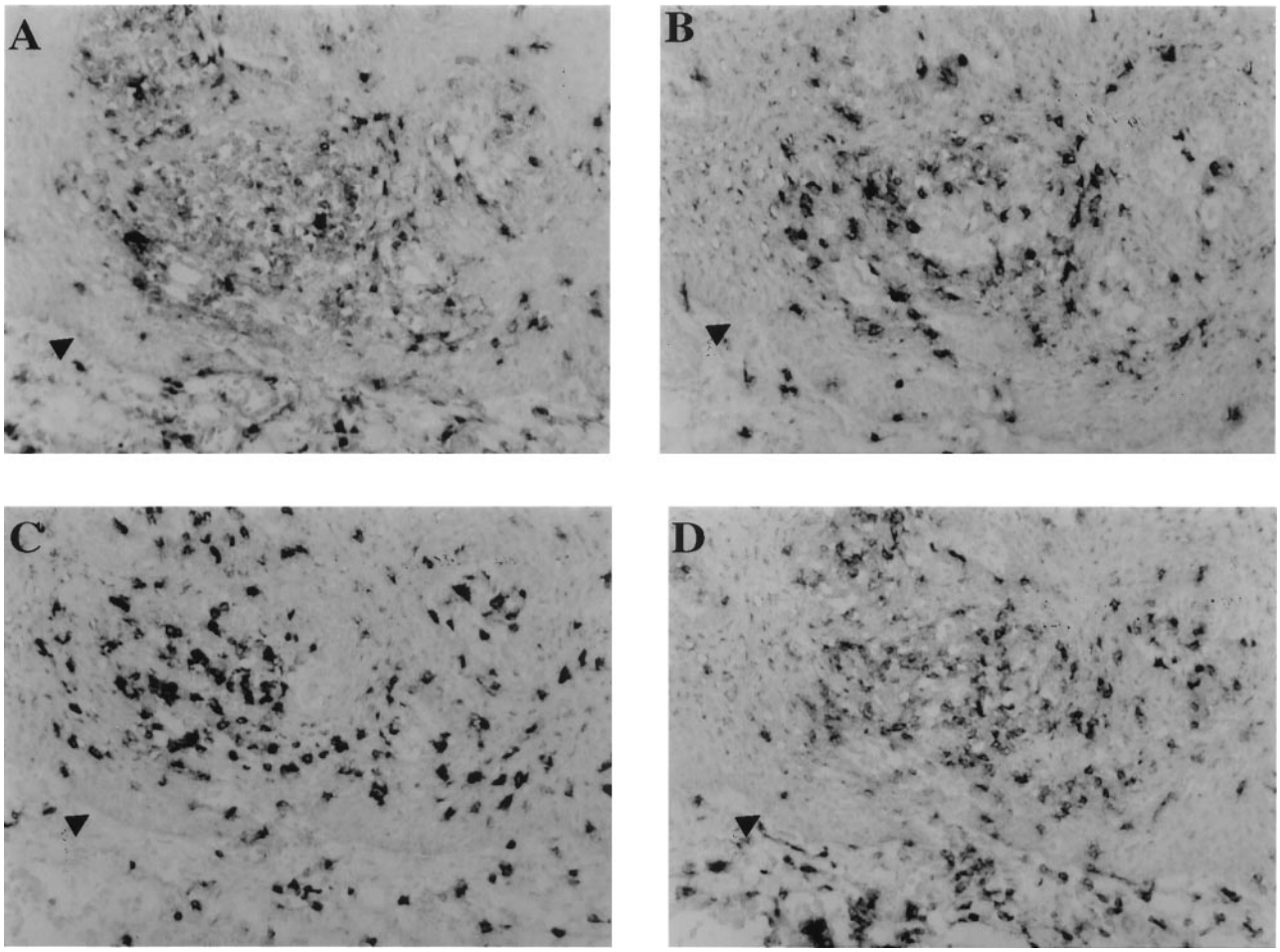

FIG. 4. Lymphocyte subtypes in intraepithelial clusters of papilloma 331/P1. Shown are serial sections stained with anti-CD4 MAb CC30 (A), anti-CD8 MAb CC63 (B), anti-WC1(γδ) MAb CC15 (C), and anti-IL2-R MAb ILA-111 (D). Magnification is ×100. The arrowheads point to the basal layer in all panels.

inent in the mucous epithelia, including that of the alimentary canal. They appear to have multiple roles (17): they can recognize antigen independently of major histocompatibility complex restriction (25), appear to be involved in delayed-type hypersensitivity responses (7), provide surveillance functions against microbes (14), and recently have been implicated in the maintenance of epithelial integrity (18). In regressing BPV-4 papillomas, γδ($WC1^+$) lymphocytes are found in even larger numbers than $CD8^+$ cells and in the same location, although they do not appear to migrate as far into the papilloma fronds as the latter. They are also more numerous than $CD8^+$ lymphocytes in noninfected palate. It is not known what their role may be in papilloma regression, but their recruitment into regressing lesions suggests that they may take part in the regression process, as suggested also by Hall et al. (9) in the case of bovine skin papillomas regressing after intralesional immunotherapy. Further investigation is needed to establish this point.

Larger clusters of lymphocytes are often found in the subbasal derma; all three subtypes of lymphocytes are found in the clusters, but intriguingly, $CD4^+$ cells, the most numerous, appear to position themselves in the middle of the cluster, whereas $CD8^+$ and γδ($WC1^+$) cells position themselves at the periphery. The significance, if any, of this is unknown. In three cases of the present series of regressing papillomas, the integrity of the basal cell layer appeared destroyed and the lymphocyte cluster, including $CD4^+$ cells, had penetrated into the suprabasal layers of the papilloma. This situation may represent one of the last stages of tumor regression. It is interesting that as long as the basal layer maintains its integrity, very few $CD4^+$ lymphocytes are found within the keratinocytes, but once the basal layer is breached, they migrate into the papilloma in large numbers. The stimuli that trigger the clustering of lymphocytes in the subbasal space and the infiltration of the papilloma are unknown.

The lymphocytes present in the papillomas are $IL2\text{-}R^+$ and are therefore activated. Whether they are activated by viral antigens, and if so which one, or by cellular antigens is not known. Heavy infiltration of lymphocytes has been observed in both BPV-2 and BPV-4 papillomas regressing after vaccination with the viral proteins L2 and E7, respectively (2, 16). In E7-vaccinated calves, T lymphocytes showed a proliferative response to the E7 antigen when challenged in vitro, both before and during papilloma regression (20), suggesting that E7-specific T cells played a role in the regression process. In that study, it was also found that in control nonvaccinated animals, E7-specific T lymphocytes were detected only at the last stages of papilloma development, before natural regression, again suggesting an involvement of E7-specific T cells in papilloma regression.

Further analysis needs to be conducted to determine whether the immunological mechanisms of natural rejection are sim-

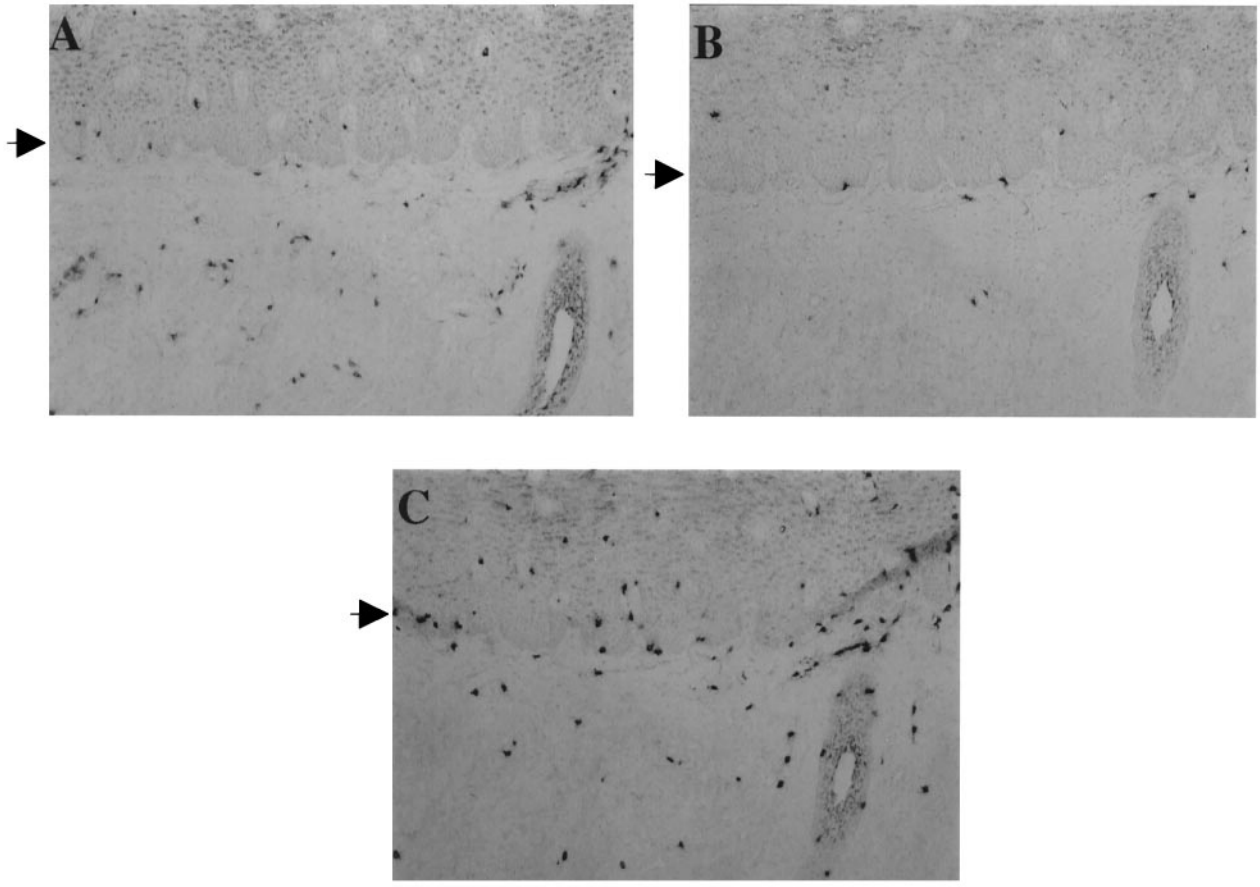

FIG. 5. Lymphocyte subtypes in noninfected palate. Shown are serial sections stained with anti-CD4 MAb CC30 (A), anti-CD8 MAb CC63 (B), and anti-WC1(γδ) MAb CC15 (C). Magnification is ×100. The arrows point to the basal layer.

ilar to those in vaccine-induced rejection. Our results for naturally regressing papillomas and those of Hall et al. (9) for immunotherapy-treated regressing papillomas suggest that this could indeed be the case. This point, however, is still to be established, as is whether the same antigens are responsible in both cases.

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