Podofilox-Induced Regression of Shope Papillomas May Be Independent of Host Immunity

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We tested the hypothesis that infiltrating leukocytes might contribute to papilloma destruction following podofilox treatment. New Zealand White (NZW) rabbits were inoculated with cottontail rabbit papillomavirus (CRPV) onto abraded areas of the dorsal skin. At 21 d after viral inoculation, 5.0% podofilox solution was applied to some papillomas, whereas others were used as controls. Three rabbits were sacrificed at each of three different periods after treatment initiation (1, 4, and 7 d). Four monoclonal antibodies (MoAbs), RG-16 (for B cells), L11/135 (specific for T cells), 2C4 (specific for class II antigen), and Ki67 (specific for proliferating cells), were used in an immunohistochemical study. All positive cells and total cells in the field were counted with an ocular grid. After 1 d of treatment, proliferation of papilloma cells was strongly suppressed in treated papillomas, but leukocytic infiltration was not altered. At 4 d and 7 d of treatment, there were substantial increases (about two to three times) in the numbers of B and T cells and class II–expressing leukocytes. The upper layers of the papillomas were highly necrotic and cell proliferation was absent in all layers. These data support the view that podofilox has a direct toxic effect on papilloma tissue. Leukocyte infiltration is not strongly associated with papilloma tissue and may not contribute to papilloma destruction. Key words: podofilox/Shope papillomas/leukocyte infiltration. *J Invest Dermatol 101:852–857, 1993*

Podophyllum resin has been used for the treatment of genital warts since the 1940s. This crude lignan is extracted from *Podophyllum peltatum* or *emodi.* This resin contains several active constituents, and podofilox may be the most active component [1,2].

Studies have reported that topical podofilox application can be used safely and effectively for home treatment, with acceptable side effects that are local and temporary [2,3]. Recently, we established a useful animal model, the Shope rabbit papilloma, for drug testing, and confirmed that podofilox is effective in the Shope papilloma system [4]. Previously, we showed that spontaneously regressing Shope papillomas had marked leukocyte infiltration in the dermis [5], and those leukocytes consisted mainly of class II–positive T cells, and few B cells [6]. The host immune system, especially T cells, plays a crucial role in spontaneous papilloma regression and leukocytes could contribute to podofilox-induced regression. In the current studies, our aim was to examine, immunohistochemically, the mechanism of topical podofilox action. Specifically, we analyzed papilloma cell proliferation and leukocytic infiltration in podofilox-treated animals.

MATERIALS AND METHODS

Animals and CRPV Nine New Zealand White rabbits (1.8–2.5 kg) of both genders were obtained from Hazelton Research Animals (Denver, PA) and housed according to appropriate guidelines in stainless steel cages and fed laboratory chow with periodic fresh kale. Cottontail rabbit papillomavirus (CRPV) was obtained from wild cottontail papilloma extracts, and stored at −70°C with no appreciable loss of infectivity since 1968 [5]. Rabbits were inoculated at two anterior sites (L1 and R1) with a 10^{-1} dilution in phosphate-buffered saline (PBS) of viral stocks and at two posterior sites (L2 and R2) with a 10^{-2} dilution onto an abraded area (1 × 1 cm) on the dorsal skin. Left-side papillomas (L1 and L2) were treated with a 5.0% podofilox solution (Oclassen Pharmaceuticals) delivered with a pipette in 500 μl, while right-side papillomas (R1 and R2) were left untreated and used as negative controls. At 21 d after virus inoculation, the podofilox treatment was started with the left-sided papillomas being treated twice daily (once in the morning and once in the evening). Nine rabbits were separated into three groups (three rabbits each group) for the different durations of treatment, and three rabbits in each group were sacrificed after 1 d, 4 d, and 7 d of treatment.

Monoclonal Antibodies L11/135 (recognizing pan T-lymphocytes [7] and 2C4 (specific for rabbit class II antigen) [8] hybridoma cells were obtained from American Type Culture Collection, TIB188 and CRL1760, respectively, and cultured in RPMI 1640 (1% fetal calf serum, 200 mM glutamine, 25 mM HEPES, 100 units of streptomycin and penicillin) for 4–5 d. 2C4 antibody was isolated from culture supernatant using hydrophobic interaction chromatography (antibody purification agarose type 1, Calbiochem Co., San Diego, CA) and used as a dilution of 1:500 with 4% bovine serum albumin in PBS. The culture supernatant of L11/135 hybridoma cells was used directly as a 1:4 dilution with 4% bovine serum albumin (BSA) in PBS. Two mouse monoclonal antibodies, RG-16 (specific for rabbit immunoglobulin [Ig]M, [Ig]G, and [Ig]A), and Ki67, specific for nuclear antigen and only present in proliferating cells [9], were purchased from Chemicon, Temecula, CA, and Dakopatts, Santa Barbara, CA, respectively.

Tissue Preparation One papilloma from the L1 site taken at 4 d and 7 d after the onset of treatment could not be used for RG-16, L11/135, and 2C4 MoAb staining because of insufficient tissue mass. Three papillomas from the other sites were examined with the three MoAbs. One papilloma from the L1 site and three papillomas from the R2 site taken from the 1-d treatment group were so small that we could not use the ocular grid for quantitative counting with Ki67 MoAb. In addition, one papilloma from the R2 site taken from the 4-d treatment group was also too small to be stained by the Ki67 MoAb. All other papillomas were divided into two parts, and either fixed in 10% neutral-buffered formalin or embedded in OCT compound (Miles Inc., Elkhart, IN) and snap-frozen in liquid nitrogen. Frozen tissue

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blocks were stored at −70°C until use. Formalin-fixed tissues were used for routine hematoxylin and eosin staining.

Staining Procedures Six-micron-thick cryostat sections of snap-frozen tissues were placed on silane-coated slides [10], air dried at room temperature, then fixed in cold acetone for 10 min. Afterward, the slides were incubated with non-immune 10% rabbit serum to block non-specific Fc receptor binding, and were then treated with appropriate primary antibodies, RG-16 (at a dilution of 1:1200), L11/135 (at 1:4), 2C4 (at 1:500), and Ki67 (at 1:16) for 30 min at 37°C. After incubation, the slides were rinsed and stained with an avidin-biotin complex staining method, Histostain-SP kit for mouse monoclonal antibodies, according to manufacturer's protocol (Zymed Labs., Inc., South San Francisco, CA).

Morphometry Quantification of positive cells was achieved by superimposition of a 0.25-X-0.25-mm ocular grid on the microscope field [5]. To count the cells in the dermis, the top of the grid was placed on the epidermal basement membrane so that the grid extended 250 μm below and 250 μm along the membrane. The grid was divided into five ranks in the vertical dimension so that each rank corresponded to a strip 50 μm in the vertical dimension and 250 μm in the horizontal dimension. For example, rank 1 extended from the basement membrane to 50 μm below, rank 2 extended from 50 μm to 100 μm, etc. On the other hand, to count Ki67-positive cells in the papillomas, the bottom of the grid was placed on the epidermal basement membrane so that the grid extended 250 μm in both the horizontal and vertical direction. The grid was also divided into five ranks in the vertical dimension, and rank 1′ extended from the basement membrane to 50 μm above, rank 2′ extended from 50 to 100 μm, etc. Within each rank, all positive cells labeled with each MoAb and total cells were counted at the same time, and the grid was moved laterally to non-overlapping fields. At least three fields were counted in each section. Enumeration of the cells was carried out at a magnification of 400 times, and Student t test was utilized for statistical analysis.

RESULTS

Analysis of B Cells in the Dermis After 1 d of podofilox treatment, sites L1 and L2 contained almost equal numbers of B cells and total cells within each rank from 3.0 cells to 5.1 cells and from 29.3 cells to 34.9 cells, respectively (Table I). In untreated sites, R1 and R2, of the same rabbits, each rank also showed very similar numbers of B cells and total cells to those of podofilox-treated sites. When we compared L and R sites, no significant difference could be seen in the numbers of B cells and total cells of L and R sites. In addition, when we compared values among each rank (five ranks in each site), there were also no significant differences. Thus, the infiltrating leukocytes in L and R sites were equally distributed in the dermis. However, at 4 d and 7 d, L1 and L2 sites (podofilox-treated sites) contained a larger number of B cells and total cells in each rank than those of R1 and R2 sites (control sites) (Table I, Figs 1*a* and 2*a*). There was a significant difference between L and R sites in 4-d and 7-d podofilox-treated rabbits (*c*, $p = 0.01-0.05$, *d*, $p < 0.01$) (Table I). However, there was no difference within each rank in L or R sites (five ranks in each site) (Table I).

Analysis of T Cells in the Dermis In 1-d podofilox-treated sites (L1 and L2), each site had almost an equal number of T cells and total cells per 0.0125 mm², from 3.8 cells to 6.8 cells and from 25.1 cells to 31.7 cells, respectively (Table II). In untreated sites of the same rabbits (R1 and R2 sites), almost the same number of T cells and total cells were seen as those in podofilox-treated sites.

There was no significant difference between each rank or each site. In 4-d and 7-d podofilox-treated rabbits, T cells and total cells in the podofilox-treated sites (L1 and L2) increased in numbers. In almost all ranks, there was a significant difference between L and R sites (Table II, Figs 1*b* and 2*b*) (*c*, $p = 0.01-0.05$, *d*, $p < 0.01$). However, no differences could be seen within each rank of L or R sites in 4-d and 7-d podofilox-treated rabbits.

Analysis of 2C4-Positive Dermal Cells The 2C4 monoclonal antibody detects rabbit class II antigen. It is known that this protein is expressed on B cells, macrophages/monocytes, and activated T cells [11–14], but not on the surface of normal keratinocytes [6,15]. We examined dermal cells that expressed class II antigen. The results were very similar to our data described above for B cells and T cells. In 1-d treated rabbits, the numbers of 2C4-positive cells in the

Table I. Quantification of B Cells in the Dermis

Rank[a]	1-d Podofilox-Treated		1-d Untreated		4-d Podofilox-Treated		4-d Untreated		7-d Podofilox-Treated		7-d Untreated	
	L1 (n = 3)[b]	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)
1	3.2 ± 1.2[c]/	5.1 ± 0.3/	3.2 ± 0.9/	3.6 ± 0.2/	10.1 ± 0.4[c]/	7.2 ± 0.9[d]/	4.5 ± 0.3/	4.2 ± 0.1/	9.0 ± 0.7[c]/	9.8 ± 2.3[c]/	3.8 ± 1.0/	4.0 ± 0.7/
	30.4 ± 1.9	34.9 ± 1.9	34.0 ± 2.7	33.7 ± 4.6	54.3 ± 5.3[e]	48.3 ± 1.9[e]	37.2 ± 0.6	35.8 ± 3.0	51.0 ± 3.0[f]	52.5 ± 2.4[d]	31.5 ± 9.4	37.3 ± 3.4
2	3.2 ± 0.8/	4.4 ± 0.5/	3.5 ± 0.6/	3.6 ± 0.7/	8.9 ± 0.6[c]/	7.5 ± 1.1[d]/	4.0 ± 0.7/	4.5 ± 0.6/	8.2 ± 0.5[c]/	9.2 ± 1.3[c]/	3.8 ± 0.6/	3.2 ± 0.3/
	31.8 ± 0.3	32.6 ± 2.5	34.8 ± 2.5	33.4 ± 2.9	54.4 ± 6.7[e]	49.0 ± 4.1[d]	33.4 ± 2.5	36.7 ± 3.2	51.4 ± 1.7[d]	54.0 ± 3.5	35.4 ± 2.7	36.1 ± 3.7
3	3.2 ± 0.7/	4.8 ± 0.9/	3.3 ± 0.7/	3.8 ± 0.4/	9.7 ± 2.4[c]/	7.3 ± 0.7[c]/	4.1 ± 0.3/	3.6 ± 0.7/	9.7 ± 2.4/	10.1 ± 1.7[c]/	3.7 ± 0.7/	4.3 ± 0.3/
	29.7 ± 0.3	33.2 ± 2.7	34.1 ± 0.6	37.6 ± 3.2	53.8 ± 7.8[e]	49.8 ± 5.2[d]	31.5 ± 2.3	35.6 ± 2.9	52.2 ± 0.5[e]	51.6 ± 2.9[e]	33.4 ± 3.6	34.6 ± 2.3
4	3.5 ± 0.9/	4.4 ± 0.2/	4.0 ± 1.0/	3.9 ± 0.6/	10.3 ± 2.3[c]/	7.7 ± 1.7[d]/	4.0 ± 0.6/	3.4 ± 0.5/	9.2 ± 1.2[d]/	8.6 ± 1.3[c]/	3.5 ± 0.6/	3.1 ± 0.4/
	30.2 ± 1.3	33.9 ± 3.7	37.2 ± 1.5	35.3 ± 5.2	53.4 ± 7.7[e]	51.6 ± 5.2[e]	31.3 ± 1.6	33.2 ± 1.5	53.0 ± 1.0[e]	49.3 ± 5.5[d]	33.5 ± 2.4	32.7 ± 2.6
5	3.0 ± 0.7/	4.2 ± 0.7/	3.9 ± 1.1/	4.2 ± 0.1/	8.6 ± 0.9[e]/	7.7 ± 1.3[d]/	4.2 ± 1.2/	4.3 ± 0.5/	9.9 ± 2.2[d]/	9.1 ± 0.6[c]/	3.9 ± 1.0/	3.8 ± 0.4/
	29.3 ± 2.2	34.8 ± 3.5	33.6 ± 2.3	35.6 ± 4.5	50.9 ± 6.2[d]	50.4 ± 4.9[d]	32.7 ± 1.5	35.1 ± 2.1	53.0 ± 2.7[e]	49.8 ± 6.1[d]	35.2 ± 1.8	32.0 ± 1.7

[a] See *Materials and Methods* for definitions of "Rank."
[b] n, the numbers of rabbits examined; L1, 5.0% podofilox treatment site (virus was inoculated with 10^{-1} dilution); L2, 5.0% podofilox treatment site (virus was inoculated with 10^{-2} dilution); R1, non-treatment site (virus was inoculated with 10^{-1} dilution); R2, non-treatment site (virus was inoculated with 10^{-2} dilution).
[c] The number of positive cells (mean ± SD)/total cells (mean ± SD).
[d] $p = 0.01-0.05$.
[e] $p < 0.01$.
[f] p value of the number of B cells and total cells was compared between L sites and R sites in each rank.

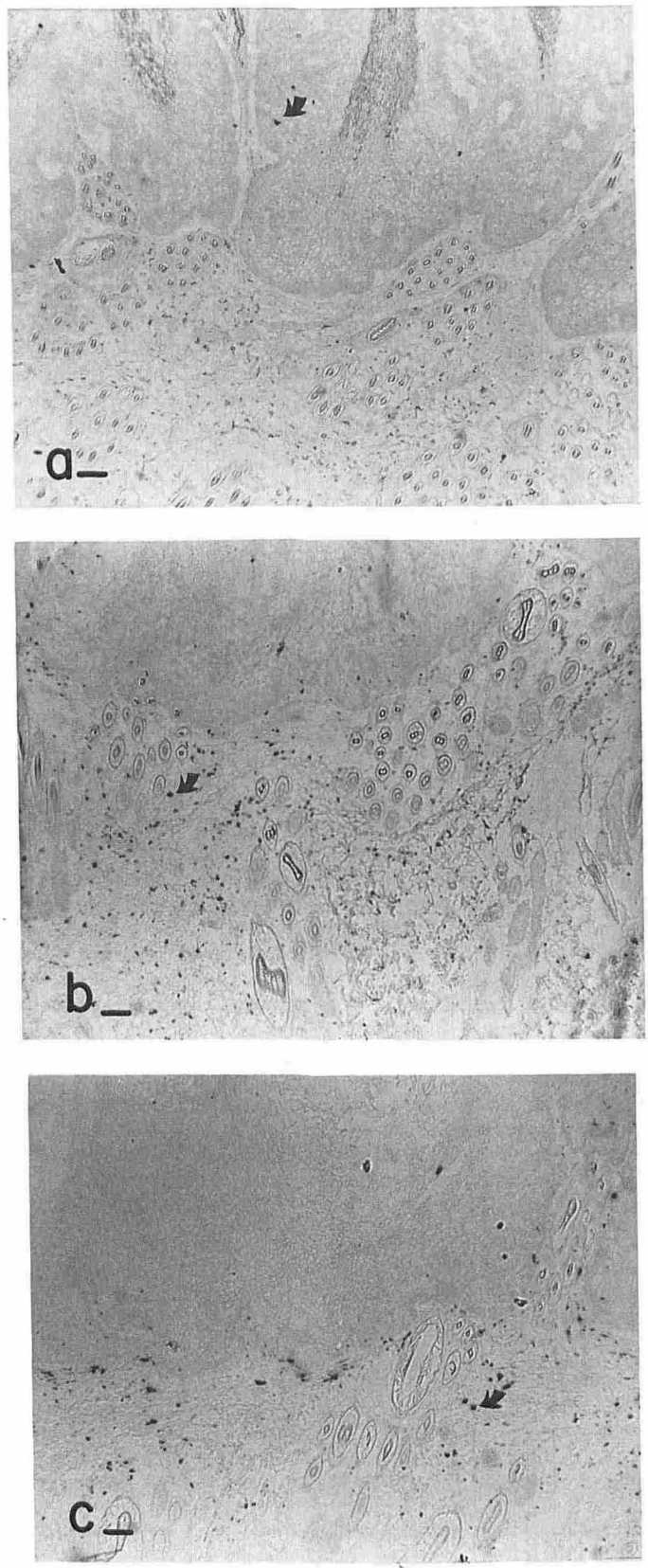

Figure 1. Immunohistochemical staining with *(a)* RG-16, *(b)* L11/135, and *(c)* 2C4 MoAbs in untreated papillomas. *Arrows, a)* representative dermal B cells stained with MoAb RG-16; *b)* representative dermal T cells stained with MoAb L11/135; and *c)* representative 2C4-positive dermal cells. *Scale bar,* 100 μm.

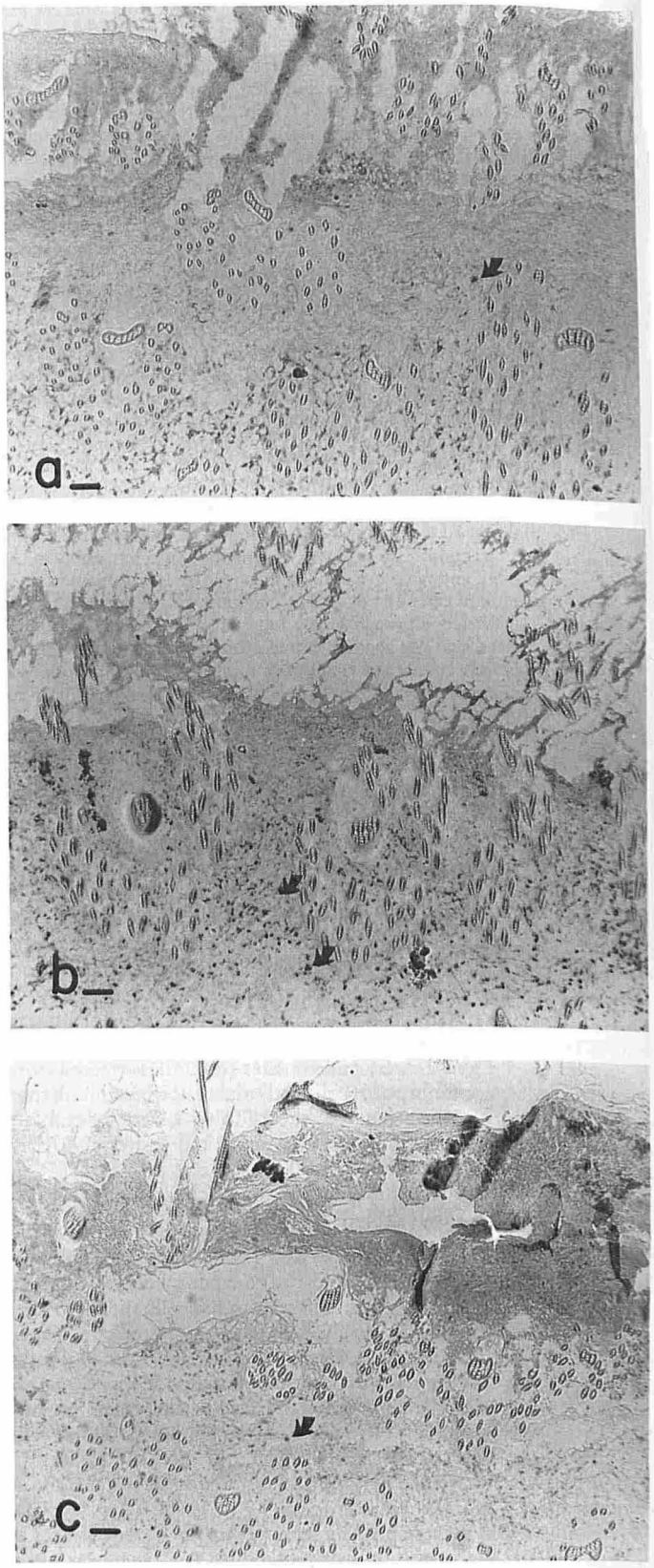

Figure 2. Immunohistochemical staining with *(a)* RG-16, *(b)* L11/135, and *(c)* 2C4 MoAbs in 4-d podofilox-treated papillomas. *Arrows, a)* representative dermal B cells stained with MoAb RG-16; *b)* representative dermal T cells stained with MoAb L11/135, and *c)* representative 2C4-positive dermal cells. Note that in podofilox-treated sites, papillomas and keratinocytes were necrotic and the border of the epidermis and the dermis was difficult to distinguish. *Scale bar,* 100 μm.

Table II. Quantification of T Cells in the Dermis

	1-d Podofilox-Treated		1-d Untreated		4-d Podofilox-Treated		4-d Untreated		7-d Podofilox-Treated		7-d Untreated	
Rank[a]	L1 (n = 3)[b]	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)
1	6.4 ± 3.0[e]/	6.1 ± 2.1/	5.8 ± 0.8/	5.9 ± 0.8/	12.9 ± 1.8[e]/	11.1 ± 0.9[e]/	5.3 ± 0.7/	6.9 ± 0.9/	7.2 ± 1.2/	8.5 ± 0.2[e]/	6.8 ± 0.4/	5.5 ± 2.7/
	28.7 ± 0.9	27.5 ± 1.1	33.8 ± 2.7	32.3 ± 4.2	55.7 ± 3.4[d]	55.8 ± 3.6[e]	38.3 ± 4.1	35.0 ± 5.2	58.5 ± 3.2[d]	52.0 ± 2.3[d]	34.5 ± 4.1	32.8 ± 6.2
2	4.7 ± 1.7/	6.8 ± 4.5/	5.1 ± 0.3/	5.4 ± 1.3/	15.2 ± 1.2[e]/	8.7 ± 1.7/	3.9 ± 0.6/	5.2 ± 1.5/	8.9 ± 0.2[d]/	9.1 ± 1.9/	7.2 ± 0.4/	4.0 ± 1.5/
	25.1 ± 2.1	28.8 ± 5.2	31.8 ± 3.5	35.5 ± 4.1	53.5 ± 0.5[d]	51.9 ± 3.8[d]	34.0 ± 5.8	30.3 ± 4.8	57.4 ± 1.4[d]	52.3 ± 0.2[d]	32.2 ± 0.8	37.1 ± 7.6
3	4.2 ± 2.1/	4.5 ± 2.4/	4.3 ± 6.2/	3.8 ± 1.3/	12.4 ± 2.4[d]	9.2 ± 1.6/	4.0 ± 0.2/	4.8 ± 1.6/	7.4 ± 0.4/	8.4 ± 1.7	6.8 ± 0.7/	3.9 ± 1.9/
	30.2 ± 1.6	31.3 ± 6.3	34.5 ± 4.7	34.7 ± 4.2	52.5 ± 1.8[d]	51.3 ± 4.4[d]	33.1 ± 7.4	32.4 ± 4.4	55.2 ± 3.9[d]	54.1 ± 1.7[e]	33.7 ± 1.7	36.1 ± 3.6
4	3.8 ± 1.7/	5.4 ± 3.3/	4.9 ± 0.8/	4.5 ± 1.1/	12.0 ± 1.7[d]/	9.7 ± 1.5[d]	5.0 ± 1.0/	4.0 ± 0.9/	6.9 ± 0.9/	7.9 ± 2.9/	5.8 ± 2.0/	4.6 ± 1.0/
	27.9 ± 1.5	30.7 ± 8.8	32.6 ± 1.9	36.3 ± 7.3	48.7 ± 1.4[d]	50.2 ± 4.6[d]	35.6 ± 9.7	35.7 ± 3.9	54.7 ± 1.4[d]	49.5 ± 3.5[d]	33.2 ± 2.7	33.8 ± 6.3
5	4.1 ± 1.8/	5.2 ± 4.6/	5.6 ± 1.6/	3.7 ± 0/	12.0 ± 2.0[d]/	9.6 ± 1.2[d]/	4.1 ± 1.7/	3.5 ± 0.9/	6.7 ± 1.7/	9.0 ± 3.2/	6.3 ± 1.7/	5.5 ± 0.7/
	29.2 ± 0.6	31.7 ± 9.7	33.5 ± 4.1	34.3 ± 6.1	53.7 ± 4.4[d]	51.9 ± 3.6[d]	34.9 ± 4.5	35.4 ± 3.0	49.9 ± 2.2[e]	50.2 ± 2.2[e]	37.7 ± 4.7	34.2 ± 7.7

[a] See *Materials and Methods* for definitions of "Rank."
[b] n, the numbers of rabbits examined; L1, 5.0% podofilox treatment site (virus was inoculated with 10^{-1} dilution); L2, 5.0% podofilox treatment site (virus was inoculated with 10^{-2} dilution); R1, non-treatment site (virus was inoculated with 10^{-1} dilution); R2, non-treatment site (virus was inoculated with 10^{-2} dilution).
[c] The number of positive cells (mean ± SD)/total cells (mean ± SD).
[d] $p = 0.01 - 0.05$.
[e] $p < 0.01$.
[f] p value of the number of T cells and total cells was compared between L sites and R sites in each rank.

Table III. Quantification of 2C4-Positive Cells in the Dermis

	1-d Podofilox-Treated		1-d Untreated		4-d Podofilox-Treated		4-d Untreated		7-d Podofilox-Treated		7-d Untreated	
Rank[a]	L1 (n = 3)[b]	L2 (n = 3)	R1 (n = 3)[f]	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)	R2 (n = 3)	L1 (n = 2)	L2 (n = 3)	R1 (n = 3)	R2 (n = 3)
1	3.9 ± 1.1[c]/	5.4 ± 2.1/	4.2 ± 1.3/	4.4 ± 0.7/	13.0 ± 2.0[d]	11.2 ± 2.5[d]	5.7 ± 0.5/	3.9 ± 0.4/	11.7 ± 2.0[d]	12.8 ± 2.6[d]	5.1 ± 1.1/	4.1 ± 0.5/
	28.7 ± 0.9	33.6 ± 10.0	38.2 ± 2.6	32.8 ± 6.4	56.5 ± 0.2[e]	51.9 ± 6.3[d]	37.0 ± 1.3	31.2 ± 6.2	47.8 ± 0.5	54.9 ± 1.4	41.7 ± 7.5	35.7 ± 3.9
2	4.0 ± 1.2/	5.2 ± 2.2/	4.0 ± 0.8/	4.4 ± 1.4/	14.0 ± 3.3[d]	10.1 ± 2.3[e]	5.9 ± 0.3/	3.6 ± 0.6/	12.7 ± 1.0[d]/	13.7 ± 2.9[d]/	5.7 ± 1.2/	3.9 ± 0.5/
	32.4 ± 8.3	36.4 ± 12.6	39.2 ± 3.9	33.6 ± 4.9	57.2 ± 1.9[e]	53.8 ± 5.7[d]	35.5 ± 2.8	28.6 ± 3.8	52.4 ± 2.4	57.0 ± 0.7[d]	35.9 ± 7.4	34.5 ± 6.5
3	4.2 ± 1.1/	5.0 ± 1.8/	4.9 ± 0.9/	3.9 ± 0.8/	13.4 ± 2.4[d]	10.9 ± 1.1[e]	4.5 ± 0.9/	3.2 ± 0.4/	13.7 ± 3.0/	13.3 ± 3.0[d]	5.0 ± 1.6/	5.6 ± 1.8/
	30.2 ± 5.3	36.4 ± 11.0	377.7 ± 4.0	35.6 ± 6.0	52.7 ± 3.0[e]	53.8 ± 5.0[e]	31.8 ± 2.5	31.3 ± 2.7	53.4 ± 0.4[d]	53.2 ± 2.8[d]	35.0 ± 5.4	32.0 ± 4.0
4	4.1 ± 1.1/	5.8 ± 1.1/	5.1 ± 2.3/	3.6 ± 2.0/	12.5 ± 3.5/	10.8 ± 1.4[e]/	4.8 ± 0.3/	3.8 ± 0.6/	11.5 ± 2.5/	11.9 ± 1.9[d]	5.8 ± 0.7/	5.0 ± 1.2/
	31.1 ± 5.2	35.8 ± 7.9	36.9 ± 4.0	36.2 ± 6.4	55.5 ± 1.2[d]	53.2 ± 3.0[d]	35.3 ± 4.7	34.7 ± 1.7	50.2 ± 0.2[d]	51.2 ± 1.1[e]	35.8 ± 2.8	32.4 ± 0.1
5	2.9 ± 0.8/	5.3 ± 1.5/	4.3 ± 0.7/	4.9 ± 1.4/	10.4 ± 1.7[d]	9.7 ± 2.4[d]	3.8 ± 0.6/	3.5 ± 0.3/	11.9 ± 3.9/	11.9 ± 1.1[d]	5.4 ± 1.8/	5.7 ± 2.3/
	29.1 ± 3.3	36.1 ± 10.6	32.7 ± 1.7	31.8 ± 7.9	50.7 ± 5.4[d]	54.0 ± 1.4[e]	29.6 ± 5.5	33.7 ± 2.7	52.7 ± 4.4	51.5 ± 1.1[d]	33.9 ± 6.5	30.3 ± 3.9

[a] See *Materials and Methods* for definitions of "Rank."
[b] n, the numbers of rabbits examined; L1, 5.0% podofilox treatment site (virus was inoculated with 10^{-1} dilution); L2, 5.0% podofilox treatment site (virus was inoculated with 10^{-2} dilution); R1, non-treatment site (virus was inoculated with 10^{-1} dilution); R2, non-treatment site (virus was inoculated with 10^{-2} dilution).
[c] The number of positive cells (mean ± SD)/total cells (mean ± SD).
[d] $p = 0.01 - 0.05$.
[e] $p < 0.01$.
[f] p value of the number of 2C4(+) cells and total cells was compared between L sites and R sites in each rank.

dermis of the L and R sites ranged from 2.9 ± 0.8 (mean ± SD) to 5.8 ± 1.1 cells and from 3.6 ± 2.0 to 5.1 ± 2.3 cells, respectively (Table III). In addition, there were almost equal numbers of total cells in each rank and each site. No significant differences could be seen in the numbers of 2C4-positive cells and total cells among each rank or each site in 1-d treated rabbits. On the other hand, in 4-d and 7-d treated rabbits, 2C4-positive cells and total cells in L sites also increased in numbers compared to the untreated sites. In nearly every rank, there were significant differences between L (treated) sites and R (untreated) sites in 4-d and 7-d treated rabbits, but no differences could be seen within each rank of L or R sites (Table III, Figs 1*c* and 2*c*).

Analysis of Ki67-Positive Cells in Dermal and Papilloma Tissues Our previous data [16] demonstrated staining of rabbit tissues with Ki67 MoAb and that the immunohistochemical staining observed was predominantly nuclear in Ki67-positive cells. Although the total numbers of cells in the L (treated) sites of 4-d and 7-d rabbits were increased, there were few cells labeled with Ki67 MoAb in the dermis as well as in all other sites (data not shown). The papillomas of L sites taken after 1 d of podofilox treatment appeared to be alive, but immunoreactivity with the Ki67 MoAb was strongly suppressed, especially in the upper layer of the papillomas. Papillomas in L sites of 4-d and 7-d treated rabbits were necrotic in all papilloma layers, and it was hard to distinguish the border between the epidermis and the dermis. However, some of those papillomas still contained intact small papillomatous regions in the marginal area of necrotic tissues. These necrotic tissues were not stained at all by Ki67 MoAb. On the other hand, all R site papillomas taken from the three different rabbit groups showed strong immunoreactivity with the Ki67 MoAb, and had a larger number and percentage of positive cells than those of the L sites in 1-d treated rabbits. Although the upper layer of the R sites (rank 4′ or 5′) had a slightly lower percentage of positive cells than the lower layer (rank 1′ or 2′), the difference was not significant. When compared with R sites, the upper layer of L sites (rank 4′ or 5′) in 1-d treated rabbits showed an extremely reduced percentage of cells labeled with Ki67 MoAb than those of the lower layer (rank 1′) or those of R sites (Table IV, Fig 3*a,b*). There was a significant difference in Ki67 MoAb-positive cells between L and R sites in 1-d treated rabbits ($p < 0.01$) (Table IV).

DISCUSSION

For over 50 years, podophyllin has long been a popular initial treatment of genital warts. Clinical studies have reported that this podofilox treatment was effective and safe for self-application at home with acceptable local side effects such as itching, burning, and erythema. At present, several techniques such as laser, cryotherapy, and surgery are used for genital wart treatment [17,18], but these methods are expensive and invasive and the recurrence still occurs. Recently, we have established an animal model system in rabbits to assess the efficacy of the podofilox treatment [4]. In this system, domestic rabbits are infected by cutaneous scarification with CRPV and, after a latency period of approximately 2–3 weeks, papillomas appear at all inoculated sites. Rapid growth of tumors continues during the next 1–2 months. In 20–30% of all hosts, the tumors do not progress further, and remain permanently benign papillomas. In approximately 10–40% of the remaining rabbits, spontaneous regression of all papillomas occurs about 1–3 months after inoculation. Primary epidermoid carcinomas develop in one or more papillomas in 40–60% of progressor rabbits [5]. Two studies have reported spontaneous regression of warts in humans [20,21]. In both human papilloma regressions and in Shope rabbit papilloma regressions, marked leukocytic infiltrates were seen in the dermis and epidermis [5]. In addition, almost all of those infiltrating leukocytes consisted of lymphocytes, mainly of T cells [6]. These results suggested that cell-mediated immunity might play a role in human and Shope rabbit papilloma regression.

We now examined the mechanism of the action of 5.0% podofilox treatment, and here compare the result with that of spontaneous

Table IV. *Quantification of Ki67-Positive Cells in the Papillomas*

	1-d Podofilox-Treated		1-d Untreated		4-d Podofilox-Treated		4-d Untreated		7-d Podofilox-Treated		7-d Untreated	
Rank[a]	L1 (n = 2)[b]	L2 (n = 3)	R1 (n = 3)[f]	R2	L1	L2	R1 (n = 3)	R2 (n = 2)	L1	L2	R1 (n = 3)	R2 (n = 3)
1′	26.2 ± 1.9[c]/	29.4 ± 6.7/	75.2 ± 4.1/	SP[g]	N[h]	N	67.8 ± 7.9/	73.7 ± 0.4/	N	N	62.4 ± 3.1/	71.1 ± 15.0/
	63.5 ± 4.2	71.6 ± 6.3	119.6 ± 6.1				108.3 ± 8.3	109.2 ± 5.5			112.2 ± 7.3	114.5 ± 8.4
	(41.3%)[d]	(41.1%)[d]	(62.9%)				(62.6%)	(67.5%)			(55.6%)	(62.1%)
2′	7.9 ± 0.9/	2.9 ± 2.4/	39.2 ± 2.4/	SP	N	N	36.6 ± 2.2/	28.0 ± 4.0/	N	N	31.7 ± 6.2/	35.5 ± 3.4/
	52.2 ± 4.9	41.2 ± 5.2	74.2 ± 4.4				60.3 ± 4.2	57.7 ± 3.0			64.4 ± 9.9	65.7 ± 8.7
	(15.1%)[e]	(7.0%)[e]	(52.8%)				(60.7%)	(48.5%)			(49.2%)	(54.0%)
3′	2.2 ± 0.1/	1.0 ± 1.2/	30.8 ± 3.8/	SP	N	N	30.8 ± 4.5/	22.7 ± 5.0/	N	N	27.1 ± 3.2/	28.1 ± 2.4/
	43.4 ± 5.4	33.8 ± 7.4	66.9 ± 6.6				55.4 ± 5.9	51.5 ± 3.5			55.9 ± 7.2	55.8 ± 8.2
	(5.1%)[e]	(3.0%)[e]	(46.0%)				(55.6%)	(44.1%)			(48.5%)	(50.4%)
4′	1.3 ± 0/	0.2 ± 0.2/	25.9 ± 2.7/	SP	N	N	28.7 ± 4.4/	19.7 ± 2.7/	N	N	22.7 ± 1.2/	25.7 ± 2.8/
	41.5 ± 7.5	26.5 ± 1.5	59.2 ± 4.5				49.8 ± 5.8	43.7 ± 2.7			39.7 ± 4.0	49.6 ± 3.2
	(3.1%)[e]	(0.8%)[e]	(43.8%)				(57.6%)	(45.1%)			(57.2%)	(51.8%)
5′	0.2 ± 0.2/	0 ± 0/	24.7 ± 5.2/	SP	N	N	24.9 ± 2.7/	19.4 ± 5.4/	N	N	21.7 ± 1.2/	20.8 ± 2.6/
	39.9 ± 7.2	24.9 ± 0.2	56.1 ± 2.0				46.6 ± 5.5	43.5 ± 3.8			49.6 ± 8.7	46.2 ± 8.3
	(0.5%)[e]	(0%)[e]	(44.0%)				(53.4%)	(44.6%)			(43.8%)	(45.0%)

[a] See *Materials and Methods* for definitions of "Rank."
[b] n = the numbers of rabbits examined.
[c] The number of positive cells (mean ± SD)/total cells (mean ± SD); ( ) = % of positive cells.
[d] $p = 0.01–0.05$.
[e] $p < 0.01$.
[f] P value of the percentage of Ki67-positive cells was compared between L sites and R1 site in 1-d podofilox-treated rabbits.
[g] SP, small papillomas (can't be measured by ocular grid).
[h] N, necrotic papillomas.

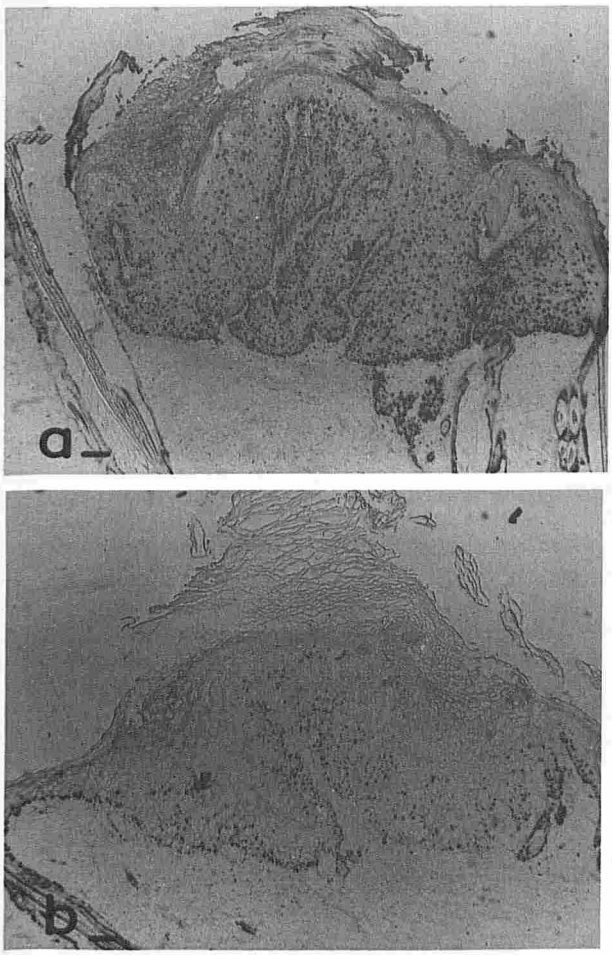

Figure 3. Immunohistochemical staining with Ki67 MoAb in untreated sites and 1-d podofilox treatment site. *a)* Untreated papillomas, *b)* 1-d podofilox-treated papillomas. Immunohistochemical staining with Ki67 MoAb in untreated site *(a)* and 1-d podofilox-treatment site *(b)*. *Arrows, (a)* representative proliferating cells in the dermis of untreated papilloma and *(b)* in 1-d podofilox-treated papilloma. *Scale bar,* 100 μm.

regression of Shope rabbit papilloma. In 1-d podofilox-treated papillomas, there was no difference in the numbers of B cells, T cells, 2C4-positive cells, and total leukocytes between treatment sites (L1 and L2) and non-treatment sites (R1 and R2). However, there was a strong difference in Ki67-positive cells in papillomas between L and R sites (Table IV). In the papillomas of 1-d, 4-d, and 7-d podofilox-treated rabbits, each rank of all R (control) sites (R1 and R2) showed very similar high percentages of Ki67-positive cells. That is, there was almost an equal number of Ki67-positive cells from the basal layer (rank 1) to the upper layer in the epidermal papillomas (rank 5). This result was consistent with our previous data examining the proliferation of progressing papillomas [6,16]. However, in 1-d podofilox-treated papillomas, L sites (L1 and L2) had remarkably decreased the percentage of Ki67-positive cells in the upper layers (ranks 3′, 4′, and 5′). This result demonstrated that the proliferation of papillomas is strongly suppressed in the uppermost layers, and that this suppression begins within 1 d of podofilox treatment. In 4-d and 7-d control papillomas, all R (control) sites (R1 and R2) had almost the same number of B cells, T cells, 2C4-positive cells, and total infiltrating leukocytes in the dermis. On the other hand, L sites of the same rabbits had an increased number of these cells when compared with R sites, and each type of cell [B cell, T cell, 2C4(+) cell, total cells] showed almost the same increasing rate. In addition, when the ratio of T cells/B cells was compared for 4-d and 7-d treated rabbits, in each rank it ranged from 0.7 to 2.0, indicating that there was an almost equal number of B cells and T cells in each rank.

As we reported earlier [6], in spontaneously regressing papillomas the number of T cells in the dermis near the basement membrane was 9 to 10 times higher than that of B cells, and most were infiltrating leukocytes concentrated near the basement membrane. In the L sites of 4-d and 7-d treated rabbits, these cells (B cells, T cells, 2C4-positive cells) were equally distributed in the dermis.

These data suggest that podofilox treatment affects papillomas both by direct toxicity to the cells and by the suppression of proliferation of any survivors. Whereas leukocytic infiltration did occur in the treatment groups, the infiltrating cells were evenly distributed throughout the dermis. This is in contrast to our findings in naturally regressing lesions, in which infiltrating cells were concentrated in specific ranks of the dermis. Furthermore, the numbers of T cells, B cells, and class II–expressing cells were roughly the same in podofilox-treated lesions, whereas T cells made up the majority of infiltrating leukocytes in our study of naturally regressing papillomas. We conclude that infiltrating leukocytes do not play the role in podofilox-induced regression that they do in naturally induced regressions and they may not play a role at all in these regressions, as evidenced by a lack of activated T-cell induction.

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