Regression of Papillomas Induced by Cottontail Rabbit Papillomavirus Is Associated with Infiltration of CD8⁺ Cells and Persistence of Viral DNA after Regression

R. SELVAKUMAR,¹ A. SCHMITT,² T. IFTNER,² R. AHMED,^{1,3,†} AND F. O. WETTSTEIN^{1,3,4*}

Department of Microbiology and Immunology, School of Medicine,¹ Jonsson Comprehensive Cancer Center,³ and Molecular Biology Institute,⁴ University of California, Los Angeles, California 90095-1747, and Institut für Klinische und Molekulare Virologie der Universität Erlangen-Nürnberg, 91054 Erlangen, Germany²

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Cottontail rabbit papillomavirus (CRPV) is a highly oncogenic papillomavirus and has been successfully used as a model to develop protective vaccines against papillomaviruses. Papillomas induced by the virus may spontaneously regress, suggesting that CRPV can also serve as a model to develop therapeutic vaccines. As a first step toward this goal, we have analyzed immunologic and viral aspects associated with papilloma regression and have identified several features unique to regression. Immunohistochemical staining of biopsies from growing and regressing papillomas and from sites after complete regression showed infiltration of CD8⁺ cells into the basal and suprabasal layers of the epidermis only during active regression. In situ hybridizations with mRNA-specific probes were strongly positive for E6 and E7 mRNAs during regression, but no late mRNA was present. Viral DNA was detected by in situ hybridization during regression but not after regression. However, analysis by PCR revealed persistence of viral DNA for several months at the majority of regression sites. The results suggest that stimulation of a strong CD8⁺ response to virus-infected cells is important for an effective therapeutic vaccine and that special attention should be given to the suppression of latent infection.

Papillomaviruses (PVs) are small DNA tumor viruses with a circular genome of about 8 kb. Replication of the viruses is highly species and tissue specific, and the viral life cycle is tightly linked to the differentiation of the stratified epithelium (38). Cottontail rabbit PV (CRPV) was the first PV identified. Its natural host is the cottontail rabbit; however, papillomas can be induced with equal efficiency in domestic rabbits. Papilloma formation in both host species is strictly limited to the hairy skin epithelium. The basis for this specificity has recently been elucidated, as it was shown that cells permissive for a high level of early viral gene expression are located in the hair follicles from which papillomas grow out (33). In papillomas induced in domestic rabbits, only low levels of mRNA encoding viral structural proteins are present (50), and little or no infectious virus can be recovered. Papillomas induced by CRPV can progress to invasive cancers (41), a property shared by a subset of genital (51) and cutaneous (31) human PVs (HPVs), the so-called high-risk HPVs. Furthermore, some papillomas induced by CRPV regress spontaneously, a fate also observed with HPV-induced lesions. Thus, in several aspects CRPV can serve as a model to study various aspects of HPV infection.

CRPV was extensively used to develop prophylactic PV vaccines. First, it was shown that both structural proteins L1 and L2 provided protection (5, 24), although immunization with L1 was superior. However, protection by L1 was provided only by the full-length, nondenatured protein (24). Particularly effective were virus-like particles produced with baculovirus vectors in insect cells (3, 6) or in yeast cells (18). Protection was also

achieved by immunization with L1-expressing recombinant vaccinia virus (25) and by DNA vaccination with an L1-expressing vector (10). Sera from L1 protein-immunized animals contained neutralizing antibodies and were effective in passive immunization (3). This suggests that protection by L1 immunization is based on neutralization of the infecting virus, and it may not involve a response to virus-infected cells. This suggestion is supported by the finding that L1-immunized rabbits are not protected against DNA-induced papilloma development. Furthermore, when infection sites of L1-immunized rabbits were probed for viral DNA by PCR 3 months after infection, no viral DNA was detected (24). Since receptors for PVs are rather ubiquitous (27), virus will be taken up by permissive keratinocytes as well as by nonpermissive cells. While permissive cells containing viral DNA may potentially be eliminated by an immune response to virus-infected cells, this is less likely the case with nonpermissive cells. Thus, the complete elimination of viral DNA in L1-immunized rabbits strengthens the notion that L1 vaccines completely prevented uptake of the virus. Effective vaccination with L1 of the natural host was also demonstrated with bovine PV types 1 (32) and 4 (4) and with canine oral PV (40).

PV infections in humans and animals usually result in a persistent infection lasting for a prolonged period of time, sometimes for the lifetime of the host. Although antibodies against structural and nonstructural viral proteins are often detected in hosts with persisting papillomas, the immune response does not eliminate the virus-infected cells. However, in humans (1, 42, 43) as well as in animals (4, 11, 19, 22, 37, 41), papillomas may regress spontaneously. In regressing human warts there was evidence of CD4⁺-T-cell infiltration (7), and the spontaneous regression of an HPV type 31 condyloma was transiently associated with an in vitro proliferation response of peripheral blood mononuclear cells (PBMCs) to an E7 peptide (20). In domestic rabbits, genetic analyses suggested that spontaneous regression was linked to certain major histocompati-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90095-1747. Phone: (310) 825-7239. Fax: (310) 206-3865.

† Present address: Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322.

bility complex class II (MHCII) allotypes, but the molecular analysis showed that it was not the MHCII gene itself but rather a gene linked to certain MHCII alleles (15, 16). Immunohistochemical analysis of spontaneously regressing rabbit papillomas indicated that regression was associated with some infiltration of leukocytes (30) and an accumulation of T cells and of cells positive for tumor necrosis factor alpha (TNF- α) mRNA (14) in the dermis below the regressing papilloma (29). Furthermore, there was a suggestion from skin tests of an increased cellular reactivity to viral structural protein in regressors (17). In vitro proliferation assays with PBMCs revealed that the regression was associated with an immune response to E2 (34). Furthermore, papilloma regression could be increased by immunization. First it was shown that vaccines prepared from papillomas increased the frequency of regression (11), and subsequently it was shown that this could be accomplished by immunization with E1 and E2 (36), the two viral proteins required for DNA replication (44), as well as by immunization with E6, the product of one of the viral oncogenes (23). These observations, together with the finding that regressor animals were resistant to infection with DNA (12), clearly indicate that regression is associated with an immune response to virus-infected cells.

Here, we have further defined steps associated with papilloma regression. It is shown that the infiltrating T cells are CD8-positive cells. During regression, expression of the early genes E6 and E7 remains at levels comparable to those in persisting papillomas. After complete regression, viral transcripts are no longer detected, but DNA persists in a latent stage for prolonged periods of time.

MATERIALS AND METHODS

Animals, virus, and biopsies. The Washington B strain of CRPV was propagated in cottontail rabbits (39) and used to infect New Zealand White rabbits obtained from Irish Farms (Norco, Calif.). Skin biopsies were obtained from infection sites 3 to 17 months after spontaneous papilloma regression or 6 to 10 months after induced regression from animals immunized with TrpE-E1 or TrpE-E2 or both (36), from normal skin and from papillomas. Biopsies were frozen in liquid nitrogen and stored at -76°C .

PCR analysis. Extracts from 4-mm punch biopsies were prepared as previously described (24, 26). Briefly, biopsies were cut into small pieces and incubated for 1 h at 55°C in 200 μl of K buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl_2 , 100 μg of bovine serum albumin per ml, 0.45% Nonidet P-40, 0.45% Tween 20, 60 μg of proteinase K per ml). The extracts were boiled for 10 min, insoluble material was removed by centrifugation, and the extracts were stored at -76°C . For DNA isolation, small biopsy pieces were incubated overnight at 55°C in 0.1 M EDTA (pH 8.0)–0.05 M Tris-HCl (pH 8.0)–0.5% sodium dodecyl sulfate–100 μg of proteinase K per ml. The DNA was then extracted twice with phenol-chloroform-isoamyl alcohol (75:24:1) for 5 min at 50°C , and phenol was removed from the aqueous phase by three successive ether extractions. NH_4 acetate was added to 0.1 M, and the DNA was precipitated with 2 volumes of ethanol at -20°C overnight. The DNA pellets were washed with 70% ethanol, dried, and dissolved in 1 mM Tris-HCl (pH 8.0)–0.1 mM EDTA. The concentration of DNA was estimated by electrophoresis in 1% agarose gels in the presence of 0.5 μg of ethidium bromide per ml together with standard amounts of uncut lambda DNA. The primer sets for amplification of different CRPV DNA segments were as follows (nucleotide numbers are according to reference 13). For the upper regulatory region (URR), the 5' primer was 5' CCTGCAC CTGTTAGTATTGTG 3', the 3' primer was 5' CTCTAGCGAGCGTGGCA GGC 3', and the amplified segment of 757 bp extended from nucleotide (nt) 7295 to 183. For E6, the 5' primer was 5' GCTGGCACTGTGTATCTGAG 3' and the 3' primer was 5' CCTGCGCACAGGATAGCAG 3', and they amplified a segment of 922 bp from nt 81 to 1002. E7 was amplified with primers as described previously (24), resulting in a segment of 344 bp from nt 1043 to 1386. For E1, the 5' primer was 5' CGCTTCCCTGGTGTGCCC 3' and the 3' primer was 5' CTGGGACTCCAACTCGTGC 3', and they amplified a segment of 1,874 bp from nt 1325 to 3198. For E2, the 5' primer was 5' GGAAG ACGAGGGTGACGATGG 3' and the 3' primer was 5' GCTGCTGATGGGA ATGGG 3', and they amplified a segment of 1,226 bp from nt 3095 to 4320. As a control for effective amplification, a primer set that amplified a fragment of rabbit cytochrome P-450 was used, resulting in a segment of 159 bp as described previously (24). The PCRs were carried out in 50- μl volumes and contained 5 μl of biopsy extract or purified DNA as indicated in Results, 50 pmol of each primer, 200 μM deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase

(GIBCO-BRL), 20 mM Tris-HCl (pH 8.4), and 100 μg of bovine serum albumin per ml. The KCl and MgCl_2 concentrations were optimized for each primer set by using the Opti-Primer Kit (Stratagene). The optimal concentrations were 1.5 mM MgCl_2 and 75 mM KCl for the URR, E1, E2, and P-450 primers and 3.5 mM MgCl_2 and 75 mM KCl for the E6 and E7 primers. Thirty cycles of amplification were performed as previously described (24) by using a Perkin-Elmer DNA thermal cycler 480. Portions (10 μl) of the PCR mixtures were electrophoresed on 1.8% agarose gels in the presence of 0.5 μg of ethidium bromide per ml. The gels were photographed, and the DNA was transferred to GeneScreen Plus (New England Nuclear) or Nytran (Schleicher & Schuell) membranes with a Turbo-blotter (Schleicher & Schuell). The membranes were hybridized with 0.2×10^6 cpm of an E7-specific probe or 1×10^6 cpm of a genomic probe, labeled by random priming with a random primer kit (GIBCO-BRL), per ml.

RNA probes and in situ hybridization. The construction of the plasmids for mRNA-specific probes and for DNA was as described previously (33). (The map of the RNA probes [antisense] is shown in Fig. 2.) The probes were synthesized in vitro in the presence of ^{35}S -UTP and had a specific activity of 3.5×10^8 cpm/ μg . The probes were reduced to a size of approximately 200 nt by alkaline hydrolysis and were tested by polyacrylamide gel electrophoresis. Sections 5 to 6 μm thick were cut with a cryostat microtome (Reichert-Jung), mounted on aminopropylsilane-coated slides, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and dehydrated through graded ethanol solutions. The sections were acetylated in 0.1 M triethanolamine–0.25% acetic anhydride for 10 min and washed in $0.2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate). The sections were then prehybridized 2 h at 42°C in solution I (45% formamide, 0.6 M NaCl, $2.5 \times$ Denhardt's solution, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.15 mg of tRNA per ml). After prehybridization, individual sections were encircled with rubber cement and hybridized with different probes in solution I containing 10% dextran sulfate. After 16 h of hybridization, the slides were washed in 50% formamide– $1 \times \text{SSC}$ at 55°C , treated with RNase A (10 $\mu\text{g}/\text{ml}$), washed twice for 2 h in $0.1 \times \text{SSC}$ at 60°C , dehydrated, and coated with Kodak NTB-2 emulsion. Slides were developed after exposure for 10 to 12 days. After staining with hematoxylin and counterstaining with eosin, the sections were evaluated with a Zeiss Axiophot microscope with a dark- or bright-field condenser. The background for RNA hybridization was determined with sense riboprobes and by pretreatment of sections with DNase-free RNase.

Staining of CD4- and CD8-positive cells. Frozen sections cut on the cryostat were air dried, fixed with acetone for 5 min at 21°C , dried again, and rehydrated in PBS. Endogenous peroxidase was quenched by incubation in 0.3% H_2O_2 . The sections were then washed twice in PBS and treated with a 1:20 dilution of normal horse serum. Biotinylated monoclonal anti-rabbit CD4 (21) and anti-rabbit CD8 (9, 49) were obtained from Spring Valley Labs. Different primary antibody batches were tested on spleen sections for proper cell surface staining. The slides were incubated with the antibody for 2 h at 21°C and then washed with PBS for 30 min. The primary signal was amplified by 1 h of incubation at 21°C with a 1:75 dilution of a mixture consisting of equal volumes of biotinylated anti-mouse immunoglobulin G and normal rabbit serum, which was precentrifuged to remove immune complexes. The sections were then washed with PBS, exposed to freshly prepared ABC reagent (Vector Labs) for 30 min at 21°C , and washed with PBS again. Color development was performed with 3-amino-9-ethylcarbazole (Sigma) and H_2O_2 in acetate buffer for 10 min. After being rinsed in H_2O , the slides were stained with hematoxylin. A monoclonal antibody to human cytokeratin 10 of the same isotype as the anti-CD4 and anti-CD8, immunoglobulin G1 kappa, served as negative control.

RESULTS

CD8 $^{+}$ -cell infiltration and viral mRNA expression in regressing papillomas. Previous investigations have shown that spontaneous papilloma regression was associated transiently with an in vitro proliferation response of PBMCs to E2 and some infiltration of the epidermis with leukocytes during regression (30). To identify immune cells potentially involved in papilloma regression and to evaluate the effect of regression on viral mRNA expression, thin sections were prepared from spontaneously regressing and growing papillomas. Immune cells were identified by immunohistochemical staining with antibodies to CD4 and CD8, and the presence of viral mRNA was assessed by in situ hybridization. A comparison between regressing and growing papillomas is shown in Fig. 1. One regressing papilloma was removed 4 months after infection, at a time when two of the four papillomas had completely regressed (Fig. 1A), and the second was removed from a regressor rabbit 6 months after infection, when three of the four papillomas had completely regressed (Fig. 1C and E); the evaluation of growing papillomas is shown in Fig. 1B, D, and F.

FIG. 1. Immunohistochemical staining of CD8⁺ cells and in situ RNA hybridization in regressing and growing papillomas. Preparation of thin sections, staining, and in situ hybridization were as described in Materials and Methods. (A, C, and E) Regressing papillomas; (B, D, and F) growing papillomas. (A and B) 100-fold magnification; (C and D) 200-fold magnification; (E and F) in situ hybridization. The general orientation of sections is with the differentiating layers of epithelium toward the top. The arrowheads point to the basal layer of the epithelium in panels A to D. Little of the dermis below the epidermis is shown. The architecture of regressing papillomas in general was less well maintained than that of growing papillomas or normal skin, and this was particularly evident with sections prepared for immunohistochemical staining which could not be fixed with formaldehyde.

Few CD8⁺ cells were present in the growing papillomas (Fig. 1B and D), while CD8⁺ cells can be seen in the basal and suprabasal layers of the regressing papillomas (Fig. 1A and C). In contrast, there was no difference in transcription pattern between the regressing and growing papillomas as revealed by a probe hybridizing to the two major early transcripts (Fig. 1E and F). The results of CD4 and CD8 staining of biopsies from normal skin, from regressing papillomas, from sites after complete regression, and from growing papillomas are summarized in Table 1, and a detailed analysis of transcription in regressing papillomas is presented in Fig. 2. The quantitative evaluation of CD8⁺-cell infiltration revealed a highly significant difference between regressing and growing papillomas. Per field of view, all three regressing papillomas contained, on the average, 90 to 100 CD8⁺ cells, while the number in growing papillomas was less than 10. No CD8⁺ cells were detected in normal skin, and sites of regression were already negative at 2 weeks postregression.

The comparison of the in situ hybridizations (Fig. 1E and F) suggested that there was no difference in viral transcripts between regressing and progressing papillomas. The more extensive analyses are presented in Fig. 2 and include analyses of a papilloma from an induced regressor. The analysis of results with mRNA-specific probes revealed a qualitative difference between transcripts in growing papillomas (33, 50) and in regressing papillomas (this work). Of the three probes specific for

late transcripts (Fig. 2, probes 6, 7, and 8), none gave a positive signal with the regressing papillomas. In this regard, there was no difference between spontaneously regressing papillomas and the papilloma of an animal in which regression was induced by immunization with TrpE-E1 (Fig. 2, rabbit 421). Since only one papilloma from an induced regressor was analyzed, it is not clear if the paucity of E1- and E2-specific transcription is related to induced regression. Overall, the immunohistochemical analyses and in situ hybridization of regressing papillomas revealed important differences from growing papillomas. The level of CD8⁺-cell infiltration was highly increased during regression. Late transcripts detected in the upper layers of growing papillomas were absent from regressing papillomas. However, the presence of CD8⁺ cells in the basal and suprabasal layers of the epidermis did not affect the abundance of early viral transcripts. Finally, within 2 weeks after complete regression, CD8⁺ cells were no longer present in the epidermis.

Persistence of viral DNA after tumor regression. In the course of our investigation of the immune response of rabbits to CRPV, spontaneous papilloma regression was observed in 24 animals, representing a regression frequency of 12%. Among the animals in which papillomas had regressed, in two animals a papilloma reemerged at one of the four regression sites. This suggested that viral DNA could persist in a latent state. To determine if DNA persistence was a rare event or

TABLE 1. Immunohistochemical staining of CD4⁺ and CD8⁺ cells in skin biopsies from growing and regressing papillomas and from postregression sites^a

Rabbit no.	Biopsy	Staining	
		CD4	CD8 (mean ± SD) ^f
344	Normal skin		
	3 days after PBS injection ^b	—	0
	11 days after PBS injection ^b	Unspecific ^d	0
380 359 359	Regressing papillomas		
	Site 4, 6 mo after infection ^c	Unspecific	99 ± 31
	Site 1, 4 mo after infection ^d	Unspecific	90 ± 20
424 350 165	Postregression		
	2 wk postregression	—	0
	3 mo postregression	—	0
435 344 345	Growing papilloma	Unspecific	8 ± 4
	Growing papilloma	—	7 ± 4
	Growing papilloma	—	6 ± 3

^a Biopsy thin sections were obtained and stained for CD4 and CD8 as described in Materials and Methods.

^b PBS was injected by high pressure into rabbit skin as described previously (33).

^c At the time the biopsy was taken, papillomas at three other sites had completely regressed.

^d At the time the biopsy was taken, papillomas on two sites had completely regressed.

^e A portion of the biopsy taken was analyzed by PCR and was still positive for CRPV DNA.

^f The staining was not associated with the cellular portion of the thin section.

^g A signal was present only in a few isolated cells.

^h For the quantitative evaluation of CD8⁺ cell infiltration into the epidermis, the numbers of CD8⁺ cells in five randomly chosen areas of approximately 1 by 0.6 mm were counted, the average number was determined, and the standard deviation was calculated.

more common, punch biopsies were obtained from regression sites and analyzed by PCR for the presence of CRPV DNA. The Southern blot analyses of regression site extracts hybridized with an E7-specific probe are shown in Fig. 3 and represent a 2-day exposure at -76°C without an amplifying screen. As can be seen, in 9 of the 15 biopsy samples an E7-specific band was detected. When the exposure was repeated for 2 days in the presence of an amplifying screen, an additional two samples (Fig. 3, lanes 7 and 15) were positive. In all samples the amplified segment of rabbit cytochrome P-450 was detected by ethidium bromide staining (data not shown). This indicated that the absence of an E7-specific band was not due to inadequate amplification of DNA in the biopsy extract. The results of the analysis of 54 biopsies are summarized in Table 2. In about two-thirds of the samples, CRPV DNA was present, and in 16 of the 19 animals tested, at least one site was positive. Furthermore, there was no difference between animals in which papillomas regressed spontaneously and those in which papilloma regression was induced by immunization with TrpE-E1 and -E2 fusion proteins (36). Finally, CRPV DNA was detected as late as 17 months after regression. These results indicate that the persistence of viral DNA after papilloma regression was a rather common feature.

The E7-specific viral band seen after Southern blot hybridization could not be detected by ethidium bromide staining, while a cellular gene-specific band could be. This suggested

that viral DNA in regression sites was present at concentrations of less than two copies per cell. To obtain information about the abundance of viral DNA, PCRs were carried out with DNA purified from biopsies by standard DNA extraction rather than with crude biopsy extracts. This permitted us to estimate the amount of cellular DNA present in PCR mixtures. The results of the analysis of the PCR-amplified DNA by Southern blotting and hybridization with a genomic probe are shown in Fig. 4. The PCR mixtures of regression site DNA contained approximately 0.15 μg of DNA (Fig. 4, lanes 1 to 5), while those of papillomavirus DNA and CRPV DNA contained 0.5 and 5×10^{-6} ng of DNA (Fig. 4, lanes 6 and 7, respectively). The additional lanes contained molecular size marker DNA (Fig. 4, lanes 8), a PCR with no DNA (lanes 9), and 0.5 μg of DNA from normal rabbit skin (lanes 10). Figure 4A shows an ethidium bromide-stained gel of PCR-amplified cytochrome P-450, and Fig. 4B and C show autoradiograms of 2- and 40-h exposures, respectively, of a filter with amplified E7 DNA. Of the five regression site samples, four were positive after a 40-h exposure. The intensity of the E7 bands at 2 h was about the same for one of the regression site DNAs and the papilloma DNA, while the CRPV DNA band was slightly weaker. Neglecting this difference, the CRPV DNA content per cell can be estimated for the regression site and the papilloma. The amount of DNA input into the PCR was 10^5 times higher for the papilloma DNA (0.5 ng) than for the CRPV DNA (5×10^{-6} ng), and the ratio of the sizes of the diploid cellular genome (6×10^9 bp) and the viral genome (8×10^3 bp) is 7.5×10^5 . Thus, on average the papilloma cells contained about 7.5 copies of viral DNA per cell. Since the amount of DNA input into the PCR for the regression site (0.15 μg) was 300 times higher than that for the papilloma PCR (5×10^{-4} μg), the regression site contained about one CRPV copy per 40 cells. A comparison of the 40-h exposure (Fig. 4C), to the 2-h exposure (Fig. 4B) suggests that the weakest positive biopsy sample contained at least 20 times less CRPV DNA. The data presented in Fig. 3 and 4 show that the abundance of viral DNA varies greatly among positive regression sites, from about one copy per 40 cells to about one copy per 1,000 cells, as some samples became positive only after a 2-day autoradiographic exposure with an amplifying screen.

The survey of regression sites summarized in Table 2 was performed by using detection of the E7 gene as an indicator. To see if retention of E7 was preferential, the additional viral genes E6 (Fig. 4E) and E1 (Fig. 4F), as well as the URR containing also the origin of replication (Fig. 4D), were amplified. As can be seen for the biopsy sample with the strongest signal for E7 (Fig. 4, lanes 5), all three other sequences tested could also be amplified, as could E2 (data not shown). After a 40-h exposure, E6 could also be detected in three of the low-copy biopsies (data not shown), but not the E1 band or the URR band. Most likely this reflects a lower efficiency of PCR amplification of these two segments. Overall, the data imply that more than the E7 gene was retained, possibly the entire viral genome.

The prolonged persistence of viral DNA at regression sites in the skin, a tissue which under goes continuous renewal, would require at least a low level of replication. If the DNA is maintained extrachromosomally, it would require expression of E1 and E2 (44); alternately, the DNA could be integrated. Because of the low abundance, a direct analysis of the state of the DNA in regression sites is not possible. The reemergence of papillomas from regression sites provided material with which the state of the DNA could be evaluated. One animal in which a papilloma reemerged may have been unusual, since it did not exhibit an antibody response to E2 before complete regression. Such a response was observed in over 80% of re-

FIG. 2. Summary of the in situ hybridization with sections from biopsies. (Top) Positions of the probes previously described (33), of the open reading frames (indicated by open boxes) (13), and of the major transcripts mapped in domestic and cottontail rabbit papillomas (28, 46). The vertical lines in the open reading frame boxes indicate the positions of the translation start sites. Dashed lines denote exons with unknown 5' boundaries, and dotted lines represent alternate splicing patterns. The early promoters are designated P1, P2, and P3, and the late promoter is designated PL (8, 47). (Bottom) Hybridization with eight antisense RNA probes and a DNA probe with sections of a regressing papilloma (site I on rabbit 421, sites I and III on rabbit 359, and site IV on rabbit 380). Adjacent serial sections were placed on one slide for hybridization with the eight different probes for RNA and the probe for DNA. +, weak hybridization; ++, moderate hybridization; +++, strong hybridization; +/-, signals in only a few isolated cells; nd, not done.

gressor rabbits surveyed (34). Two-dimensional analysis of the DNA from the reemerging papilloma of this animal is presented in Fig. 5, which shows, from lower right to upper left, the positions of monomeric, dimeric, and tetrameric supercoiled DNAs (asterisks) with larger circular DNA species moving even slower in both dimensions. Although a linear DNA trail is clearly visible, the presence of discrete spots corresponding to monomeric to tetrameric linear DNAs (small arrow heads) suggests that linear DNA was generated from random degradation of extrachromosomal DNA. At the position where the bulk of the cellular DNA migrated (large arrowhead), as visualized by ethidium bromide staining, only a small portion of the viral DNA was present, and this suggests that at least in this case CRPV DNA was maintained extrachromosomally after regression.

DISCUSSION

Here we have presented evidence for a regression-associated infiltration by CD8⁺ cells into papillomas. The difference in

CD8⁺ infiltration between growing and regressing papillomas is much more dramatic than the rather small difference observed by comparing total leukocyte infiltration (30). In the basal and suprabasal layers, where the CD8⁺ cells are present, there is no evidence of local necrosis, suggesting that the CD8⁺ cells were not massively involved in cell killing. It is likely that the CD8⁺ cells specifically recognize viral antigen presented on kercinocytes, and this would require a direct contact between the infected cells and CD8⁺ cells. This recognition may then lead to a release of cytokines. However, this does not affect viral RNA expression locally, but it may be critical to attract leukocytes into the dermis below regressing papillomas, which were shown to contain TNF- α mRNA (14). The importance of a direct cellular recognition of virus-infected cells is suggested by the finding that E1-induced regression is not always accompanied by a humoral response to E1 (36). Surprisingly, the reagents used did not reveal any infiltration of CD4⁺ cells in the epidermis. Although a technical problem specifically related to staining of CD4⁺ cells in the epidermis cannot be

FIG. 3. Southern blot analysis of PCR-amplified viral DNA from extracts of regression sites. Biopsy extraction, PCR amplification, and Southern blot hybridization with an E7-specific probe were performed as described in Materials and Methods. The PCR amplification mixtures contained the following: lane 1, no DNA; lane 2, extract from normal skin; lanes 3 and 19 molecular size markers; lanes 4 to 7, extracts from spontaneous regression sites; lanes 8 to 18, extracts from induced regression sites; lane 20, 0.5 ng of papilloma DNA. The position of the E7-specific band is indicated by an arrowhead on the left and those of the markers in the marker lanes and their sizes (in base pairs) are given on the right. The exposure shown was for 2 days at -76°C without an amplifying screen. After a 2-day exposure with an amplifying screen, E7 bands were also detected in lanes 6 and 14 but not in lanes 8 and 9.

ruled out, this appears unlikely, since CD4^{+} -cell staining was strong when tested on spleen sections.

The cytotoxic T-cell response inducing papilloma regression appears to be relatively weak compared to a response in a systemic viral infection. This is indicated by the finding that a specific cytotoxic T-cell response could be demonstrated with PBMCs from vaccinia virus-infected rabbits but not with PBMCs from regressor rabbits (35). The data thus suggest the following events in papilloma regression. Virus-infected cells are recognized by infiltrating CD8^{+} cells. The effect of CD8^{+} cells on papilloma development is strongly amplified by $\text{TNF-}\alpha$ -secreting leukocytes attracted by CD8^{+} cells to the dermis below papillomas. This results in abrogation of the virus-stimulated growth in the suprabasal layer (14), directly or indirectly eliminates late viral gene expression, and results in necrosis in the upper layer through apoptosis (14).

Regression, surprisingly, does not result in elimination of all cells which harbor viral DNA. One possibility is that the DNA in these cells was integrated. Clearly, the two-dimensional gel analysis of the DNA from a papilloma which reemerged from a regression site showed that it contained almost exclusively extrachromosomal DNA, and this strongly suggested that the DNA was maintained extrachromosomally after regression. It is unlikely that the DNA changed from an integrated to an extrachromosomal state as cells became competent for papilloma development. Although such a process can be inferred from experiments with simian virus 40 where cocultivation of transformed cells with permissive cells resulted in the recovery of infectious virus, the process seems to be quite inefficient (45). No such observations have been reported for PVs, where the opposite process is often observed. The findings that spontaneous regression is associated with an immune response to

FIG. 4. PCR amplification of different segments of the early CRPV region of DNA isolated from biopsies and CRPV. DNA was isolated from regression site biopsies from a papilloma and from CRPV. DNA isolation, PCR amplification of the different segments, and Southern blot hybridization with a genomic probe were as described in Materials and Methods. Autoradiography was for 2 h (B, D, E, and F) or 40 h (C). (A) Ethidium bromide-stained gel shown also in panel D. The primers present in the PCR were for P-450 and the URR (A and D), E7 (B), E6 (E), and E1 (F). The PCR mixtures contained about $0.15\text{ }\mu\text{g}$ of DNA isolated from regression sites (lanes 1 to 5), 0.5 ng of papilloma DNA (lane 6), or $5 \times 10^{-6}\text{ ng}$ of CRPV DNA (lane 7). Molecular size markers were present in lane 8, and the positions and sizes of the markers (in base pairs) are indicated on the right. Lane 9, PCR without DNA; lane 10, PCR with $0.5\text{ }\mu\text{g}$ of normal rabbit skin DNA. The positions of relevant PCR products are indicated by arrowheads on the left.

E2 (34) and that immunization with E1 and E2 (36) as well as with E6 (23) greatly increases the regression frequency suggest that the cells surviving regression do not express high levels of these viral proteins. Since both E1 and E2 are required for PV DNA replication (44), the residual DNA may reside in cells which are in a resting stage. Attempts to directly identify viral mRNA by reverse transcription-PCR in biopsies from regression sites were negative. A regression site with a relatively high DNA content (one viral gene copy per 40 cells) was compared to a growing papilloma (75 viral DNA copies per cell). The negative results for the detection of E6 and E7 mRNAs suggested that viral transcription relative to the DNA content at the regression site was at least an order of magnitude lower than that in the papilloma (35). Continued production of some viral protein by CRPV DNA-containing cells after regression, however, was suggested. When biopsies of regression sites and normal skin were explanted into interleukin-2-containing medium, CD8^{+} cells were detected by fluorescence-activated cell sorter analysis only in lymphocytes growing out of regression site biopsies. Since CD8^{+} cells were not detected in the epi-

TABLE 2. Persistence of viral DNA after spontaneous and induced papilloma regression^a

Regression	No. with DNA detected/total no. (%)	
	Sites	Animals
Spontaneous ^b	14/22 (64)	7/9 (78)
Induced ^c	20/32 (62)	9/10 (90)

^a PCR was performed with extracts from regression sites, and viral DNA was detected by Southern blot hybridization as described in Materials and Methods.

^b Biopsies were obtained 6 to 20 months after infection or about 3 to 17 months after regression.

^c Biopsies were obtained 9 to 13 months after infection or about 6 to 10 months after regression from animals immunized with TrpE-E1 or -E2 or both before infection.

FIG. 5. Two-dimensional agarose gel analysis of DNA from a papilloma which reoccurred at a regression site. An untreated rabbit infected with CRPV at four sites developed papillomas at all four sites. Two months after infection, all four papillomas had regressed. After an additional 2 months, a papilloma started to reemerge at one side and was removed a year later. No papillomas developed at the three other regression sites. The DNA was isolated essentially as described for DNA isolated for PCR (see Materials and Methods). Two micrograms of DNA was electrophoresed overnight in 0.5% agarose in the first dimension and again overnight in 1.0% agarose in the second dimension. Southern blot preparation and hybridization with a genomic probe were as described for PCR samples. The position of the bulk cellular DNA visualized with ethidium bromide staining is indicated by the large arrowhead. The identification of linear CRPV DNAs (small arrowheads) and of supercoiled DNAs (asterisks) is based on previous analyses (48). The asterisks, from lower right to upper left, indicate the positions of supercoiled monomeric, dimeric, and tetrameric DNAs. The small arrowheads, from lower right to upper left, indicate the positions of linear monomeric, dimeric, trimeric, and tetrameric DNAs.

dermis by immunohistochemistry after regression, the outgrowing CD8⁺ cells may have resided in the dermis. Alternatively, their number may have become significant only after growth stimulation with interleukin-2. That cells containing latent CRPV DNA may become competent to express viral genes at a high level is indicated by the spontaneous reemergence of papillomas after regression and by the finding that in rabbits infected with a low dose of virus which did not permit papilloma development, papilloma growth could be induced by skin irritation (2). Finally, it is possible that after macroscopic regression there is a continuous equilibrium between the immune response killing virus-infected cells and virus-infected cells escaping elimination. At present, we cannot exclude this possibility. However, it is less likely, since immune suppression with cyclosporine of two CRPV DNA-positive regressor rabbits did not result in a reemergence of papillomas (35).

Our experiments have provided new insights into papilloma regression, with important implications for therapeutic vaccine development. It appears that it will be critical to elicit a strong CD8⁺-cell response, and special consideration has to be given to the prevention of latent infection.

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