T-Cell Response to Cottontail Rabbit Papillomavirus Structural Proteins in Infected Rabbits

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Cottontail rabbit papillomavirus (CRPV)-induced papillomas progress at a high frequency to carcinomas and thus can serve as a model for high-cancer-risk human papillomavirus infection. Previously, we have shown that antibodies to nonstructural and structural proteins are detected in only a fraction of papilloma-bearing animals. However, the antibody response to structural proteins drastically increases as papillomas progress to carcinoma (Y.-L. Lin, L. A. Borenstein, R. Selvakumar, R. Ahmed, and F. O. Wettstein, J. Virol. 67:382–389, 1993). Here we have monitored the cellular immune response to viral proteins during the course of infection and particularly during progression from papilloma to carcinoma. This was done by measuring the in vitro proliferation response of peripheral blood mononuclear cells (PBMCs) to CRPV structural proteins L1 and L2. The proliferating cells were identified as T cells by selective removal of B or T cells. In general, the T-cell response was low for rabbits at the papilloma stage and none responded to L2. Lymphocytes from animals with carcinomas more frequently and more strongly responded to L1, and more than half also responded to L2. In addition to stimulation of PBMCs, L1- and L2-specific proliferation could also be demonstrated with lymph node and spleen cells. Overall, our data show that progression of papilloma to carcinoma is associated with an increased T-cell response to CRPV structural proteins in addition to an increased humoral response. This greater immune reactivity, however, was not associated with a selectively increased expression of structural proteins, since RNA isolated from papillomas and carcinomas contained similar relative levels of late and early RNA as shown by dot blot analysis. Thus, the heightened immune reactivity seen in carcinoma-bearing rabbits most likely reflects greater stimulation of the immune system owing to dissemination of the tumor. These findings suggest that increased immune responses to papillomavirus proteins may be prognostic of progression to carcinoma and particularly of the development of metastases.

Papillomaviruses are small DNA viruses with a circular double-stranded DNA of about 8 kb. The viruses are highly species specific, and viral replication is confined to the epithelium and linked to the differentiation of epithelial keratinocytes (21). Most papillomavirus infections result only in local lesions characterized by growth stimulation of the infected epithelium, leading to the formation of papillomas or warts. Papillomas can persist for a very long time, often the lifetime of the host, and this suggests that these viruses can evade host defense mechanisms effective against other viruses. The spontaneous regression of papillomas and the increased development of papillomavirus-induced lesions in immunosuppressed hosts (8), however, suggest that the immune system can affect the host-virus interaction. A major interest in papillomaviruses derives from the fact that lesions induced by some of these viruses progress to carcinoma. Thus, certain genital human papillomaviruses (HPVs), such as HPV type 16 (HPV16), HPV18, and others (25), play an essential role in the development of anogenital cancer, and some cutaneous HPVs, e.g., HPV5 and HPV8, are associated with skin cancers in patients with the relatively rare disease epidermodysplasia verruciformis (12).

The first papillomavirus isolated was the cottontail rabbit papillomavirus (CRPV) (15), and it is also the first papillomavirus for which a connection between papillomavirus infection and cancer was clearly established (14). In the natural cottontail rabbit host, about 25% of infected animals develop cancers. Domestic rabbits are equally susceptible to viral infection, but produce little if any virus. However, papillomas on more than two-thirds of the animals progress to cancers (20). In both species, a portion of the infected animals have papillomas that spontaneously regress; regression in domestic rabbits was shown to be linked to a major histocompatibility complex class II allotype (3). Furthermore, in animals with regressing papillomas there was increased leukocyte infiltration in the dermis and most of the cells were T cells (10, 11).

Previously, we analyzed the humoral immune response to CRPV proteins in virus-infected rabbits (6). At the papilloma stage, less than a third of the animals were positive for any of the viral proteins, using Western immunoblots, and none reacted with E4, E5, and L2. After progression to carcinoma, there was a drastic increase in the frequency and intensity of the response to the two viral structural proteins L1 and L2. In this investigation we have examined the T-cell response to the two viral structural proteins in animals with papillomas and carcinomas.

In vitro proliferation assay for rabbit PBMCs. To further explore the basis for the change in the immune response to structural proteins during progression, the cellular immune response to these proteins was evaluated. This was accomplished by developing an in vitro proliferation assay for rabbit peripheral blood mononuclear cells (PBMCs). Two carcinoma-bearing rabbits were chosen, one with a strong antibody
response to L1 and one with a strong response to L2, as determined by Western blotting as described previously (6). PBMCs were isolated from 60 to 70 ml of blood obtained from the ear artery and collected into heparinized tubes. The blood was diluted 1:1 with phosphate-buffered saline (PBS), layered onto an equal volume of Histopaque-1.077 (Sigma Chemical Co.), and centrifuged at 2,400 rpm for 12 min in an IEC 210 rotor. The cells at the interphase were collected and washed three times in PBS and once in minimum essential medium. The viability of the purified cells was greater than 95% as determined by exclusion of trypan blue, and the concentration was adjusted to 2 x 10^7 cells per 100 μl of culture medium consisting of RPMI 1640 (GIBCO) supplemented with 1% normal rabbit serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Standard proliferation assays were performed in 96-well flat-bottom microtiter plates. To each well, 100 μl of cell suspension was added and 100 μl of antigen solution containing fusion protein trpE-L1, trpE-L2, or trpE prepared as described previously 5 at 1 to 3 μg per ml. Then, the cultures were incubated at 37°C in 5% CO₂-95% air for 2 to 8 days. Additional wells contained cells and medium only or cells and 5 μg of concanavalin A per ml. Twenty hours prior to harvest, 1.0 μCi of [3H]thymidine (specific activity, 2 Ci/mmol [New England Nuclear Corp., Boston, Mass.]) in 25 μl of medium was added. Duplicate wells were harvested for each time point, and the average amount of [3H]thymidine incorporated was determined. The results of the [3H]thymidine incorporation experiments are presented in Fig. 1. The trpE-L1- and trpE-stimulated incorporation into PBMCs of the L1-responsive rabbit is shown in Fig. 1A, and the trpE-L2 and the trpE stimulation for the L2-responsive rabbit is shown in Fig. 1B. Maximal incorporation was observed after 4 to 7 days, and strong positive responses were also detected by the presence of blast cells in the plate wells. The amount of [3H]thymidine incorporated in response to trpE was considerably lower than that in response to the fusion proteins but still was higher than that incorporated into cells incubated in the presence of medium only, which was usually around 100 cpm (data not shown). As can be seen, the incorporation into PBMCs from an uninfected animal cultured in the presence of trpE or trpE-L1 was not significantly different at any time point (Fig. 1A). To provide a numerical value for the specific stimulation, a stimulation index (SI) was calculated, which represents the ratio of the highest amount of [3H]thymidine incorporated in wells containing trpE-L1 or trpE-L2 divided by that incorporated into wells with trpE measured on the same day. On day 6, the SI for L1 (Fig. 1A) was 5.3, and that for L2 (Fig. 1B) was 3.4. SIs greater than two are considered positive responses to the viral proteins.

Characterization of proliferating cells. Reagents specific for rabbit T and B cells were used to identify the cells proliferating in response to CRPV structural proteins. PBMCs from animals with a positive proliferation response to L1 were incubated with anti-rabbit thymocyte serum (RTLA; Cederlane) or normal medium for 1 h on ice, washed, and incubated with complement (Cederlane). After complement treatment, the cells were washed in serum-free RPMI 1640 and resuspended in complete culture medium at 2 x 10^5 cells per 100 μl. The recovered cells were then tested in a standard proliferation assay, and the results of a representative experiment are shown in Fig. 2A. Treatment of cells with anti-rabbit thymocyte serum and complement greatly reduced concanavalin A-stimulated [3H]thymidine incorporation compared with that of control cells treated with normal medium and complement (Fig. 2A, left graph). This indicated that a high portion of the T cells were removed. The treatment also reduced greatly the trpE-L1-stimulated incorporation (Fig. 2A, right graph).

![Figure 1](http://jvi.asm.org/)

**FIG. 1.** In vitro [3H]thymidine incorporation into PBMCs of CRPV-infected domestic rabbits in response to stimulation with trpE-L1 (A) and trpE-L2 (B). The amount of [3H]thymidine incorporated into PBMCs was measured during a 20-h pulse at different days of in vitro culture in the presence of antigen as described in the text. (A) PBMCs from a CRPV-infected rabbit that was positive for L1 on Western blot were cultured in the presence of trpE-L1 (filled squares) or trpE (open squares); PBMCs from a control rabbit were also cultured in the presence of trpE-L1 (filled circles) or trpE (open circles). (B) PBMCs from a CRPV-infected rabbit that was positive for L2 on Western blot were cultured in the presence of trpE-L2 (filled triangles) or trpE (open triangles).

![Figure 2](http://jvi.asm.org/)

**FIG. 2.** Effect of B- and T-cell depletion from PBMCs on the L1-specific proliferation response. (A) PBMCs were treated with anti-rabbit thymocyte serum and complement, depleting the T cells, or with medium only and complement as described in the text. The amount of [3H]thymidine incorporated was measured after stimulation with concanavalin A (left graph) or with trpE-L1 or trpE (right graph) in a standard assay as described in the text. The values for the day with the highest stimulation are given. Incorporation into anti-rabbit-thymocyte serum plus complement-treated PBMCs (filled bars); incorporation into medium plus complement treated cells (open bars). (B) B cells were selectively removed by binding to anti-rabbit IgG-coated petri dishes, and control cells were exposed to uncoated petri dishes as described in the text. PBMCs depleted of B cells and control cells were stimulated with anti-rabbit IgG, trpE-L1, or trpE in a standard proliferation assay. The amounts of [3H]thymidine incorporated are given for the days with the highest values. Control cells (filled bars); B-cell-depleted PBMCs (open bars).
results were obtained in four different experiments, which suggested that T cells represented the major PBMC component responding to the viral structural protein.

To further strengthen this conclusion, the effect of B-cell removal on antigen-stimulated proliferation was evaluated. B cells were removed by binding to anti-rabbit immunoglobulin G (IgG)-coated petri dishes. Petri dishes (100-mm diameter) were coated with 50 μg of affinity-purified goat anti-rabbit IgG (Cappel Laboratories). Purified PBMCs (1 × 10⁷ to 2 × 10⁷) were added to coated or uncoated petri dishes and incubated at 4°C for 2 h. Nonadherent cells were recovered by gentle washing with serum-free RPMI 1640 medium and stimulated in a standard proliferation assay with 100 ng of goat anti-rabbit IgG, 60 ng of trpE-L1, or 60 ng of trpE per well (Fig. 2B). The effective removal of B cells by binding to anti-rabbit IgG-coated petri dishes is demonstrated by the great reduction in [³H]thymidine incorporation in response to stimulation with anti-rabbit IgG. The depletion of B cells was also confirmed by fluorescence-activated cell sorter analysis (data not shown). The trpE-L1-stimulated [³H]thymidine incorporation, however, is higher in the T-cell-enriched nonadherent cells than in control cells, whereas trpE-stimulated incorporation is low with both. Overall, these data demonstrate that T cells specifically responded to L1.

**Proliferation response to L1 and L2 during progression to carcinoma.** To determine whether an increased recognition of viral structural proteins with tumor progression was also a feature of the cellular immune system, PBMCs from papilloma- and carcinoma-bearing animals were tested for their in vitro proliferation response to trpE-L1 and trpE-L2 and to trpE. The SIs were calculated as described above to provide a measure for the specific response to L1 and L2. The values shown in Fig. 3 are the highest ones for each rabbit and tumor stage. SIs greater than two were considered positive. Seventeen rabbits with papillomas were tested for their responses to L1 and L2. Eight rabbits had a positive response to L1 (SI > 2), but the majority of the responses were low (Fig. 3A, left), whereas no animal had a positive response to L2 (Fig. 3B, left). Twelve rabbits were evaluated at the carcinoma stage; 10 rabbits were tested for both antigens, and 1 each was tested for L1 or L2 only. Nine of the 11 rabbits had a positive response to L1, and in the majority the responses were medium to high (Fig. 3A, right). In contrast to the absence of a response to L2 at the papilloma stage, 7 of 11 carcinoma-bearing animals responded positively to L2 (Fig. 3B, right). In animals for which both antigens were evaluated, the response to L1 in general was higher.

In addition to PBMCs, the cellular immune response to the structural proteins was also measured in spleen and lymph node cells (Table 1). Single-cell suspensions were prepared from spleen and lymph node tissues and erythrocytes were removed from spleen cells by treatment with 0.83% NH₄Cl. The proliferation response analyses were carried out as described for PBMCs. The papilloma stage analyses were performed during the early phase of the stage, at 0.5 to 4 months after infection. Most responses were negative, but in two animals a low response to L1 was detected; in one, both PBMCs and spleen cells responded and in another, only lymph node cells were weakly positive. At the carcinoma stage, cells from a much higher percentage of animals were stimulated by viral antigens and a response was usually detected with cells from more than one tissue. Overall, the analyses clearly show a difference in the cellular immune response to viral structural proteins among rabbits at the papilloma and carcinoma stage.

**Proliferation and antibody response to viral structural proteins.** The development of the proliferation response to viral structural proteins was monitored in two animals over a period of several months (Fig. 4). In one animal (Fig. 4A), the response to L1 was low at 2 and 6 months and finally, at 7.5 months after infection, the proliferation response had drastically increased. The antibody response to L1 was not measured at 2 months, was low at 6 months, and was stronger at 7.5 months (data not shown). At 7.5 months, no overt carcinoma was detectable, but a month later carcinoma clearly had developed. This is an important point and shows that the pronounced increase in immune responses to L1 preceded the

![Graph](https://via.placeholder.com/150)

**FIG. 3.** Proliferation response to viral structural proteins L1 and L2 in rabbits at the papilloma and carcinoma stages. (A) Proliferation responses to trpE-L1 and trpE were measured in papilloma- and carcinoma-bearing rabbits, and SIs were determined for the specific response to L1 as described in the text. (B) Specific responses to L2 were determined as described for panel A. Responses are considered positive when the SIs were greater than two (dashed lines).

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* In vitro stimulations of PBMCs lymph node and spleen cells were performed, and the SIs were calculated as described in the text. SIs of <2 are not considered to represent a positive response.

* P, papilloma; C, carcinoma.

* ND, not done.
obvious clinical appearance of carcinoma. Thus, it is possible that increased immune reactivity to viral proteins may be used as a diagnostic indicator of progression to carcinoma. The proliferation response to both L1 and L2 was monitored in a second animal (Fig. 4B). The response to L1 was low at the papilloma stage, at 6.5 and 11 months after infection, and increased as carcinomas developed at 14.5 and 16 months. No antibody to L1 could be detected at 6.5 months, but a strong response was evident at 11 months and thereafter. The proliferation response to L2 was negative until after carcinomas had clearly developed. Surprisingly, and, an exception, this animal was positive for L2 on Western blot at the papilloma stage. The L1 and L2 proliferation response analyses of cells from many papilloma- and carcinoma-bearing rabbits presented here and the previously reported results of the humoral response to these antigens exhibit a similar trend. When both humoral and cellular responses were determined in the same animal at the same time, however, some divergence was evident.

**Dot blot analysis of late transcripts in papillomas and carcinomas.** Here we have shown that the progression of papillomas to carcinomas is associated with an increased cellular immune response to viral structural proteins. A similar increase in the response to the structural proteins during progression was also detected in the humoral response; however, no such increase was observed in the response to nonstructural proteins (6). The reason for this selective change is not known. In domestic rabbit tumors, the characteristic transcripts encoding L1 and L2 could not be detected in Northern (RNA) blots (9, 13, 23). The increase in both antibody and proliferation response long after infection suggests that these antigens continue to be synthesized and their synthesis may be higher in carcinomas. To determine whether the level of late mRNA in carcinomas was specifically increased relative to early mRNA, dot blots were prepared. Poly(A)⁺ RNA was isolated from papilloma and carcinoma tissues and treated with RNase-free DNase, and duplicate dot blots were prepared as described previously (7). One blot was hybridized with a probe that contained open reading frame L1 which hybridizes with L1 as well as with L2 mRNA (9) (Fig. 5A), and one blot was hybridized with a full genomic CRPV probe (Fig. 5B). Dots in rows A and B of each RNA preparation contained the same amount of RNA. The RNAs spotted in row A, however, were treated with NaOH before being spotted onto the filter. Rows C and D contained decreasing amounts of RNA. Papilloma RNA was present in rows 1, 2, and 4, and carcinoma RNA was in rows 3, 5, and 6; row 7 contained RNA from a CRPV DNA-negative rabbit epithelial cell line. The absence of spots in rows A showed that the RNA was not contaminated with any DNA. Control dot blots with NaOH or formamide-denatured viral DNA showed strong hybridization with both probes (data not shown). Although there is a considerable variation in the content of viral RNA, the relative intensity of corresponding spots between Fig. 5A and B is similar. This indicated that there was no major difference in late relative to early gene transcription between papillomas and carcinomas. The dot blot data thus suggest that the increased cellular and humoral immune responses to L1 and L2 were not related to a selective increase in the level of late gene expression.

In the analyses performed here, the cellular immune responses to viral structural proteins in papilloma- and carcinoma-bearing rabbits were determined. This was of particular interest since the investigation of the humoral response (6) showed a major change during tumor progression in the response to these proteins but not to others. First, it was demonstrated that selective removal of T cells from PBMCs abolished the response to L1, whereas removal of B cells increased the response. This demonstrated that T cells specifically responded to L1. Comparison of the T-cell responses to L1 between papilloma- and carcinoma-bearing rabbits showed an increase of from 47 to 82% with tumor progression. The previously reported increase in the humoral response to L1 was from 29 to 91% (6). With L2, the difference between the stages was more dramatic as no animals at all responded to L2 at the papilloma stage while 58% were positive at the carci-
noma stage. These differences are very similar to those observed with the antibody response for which the change was from 0 to 50% (6). Thus, both the cellular and humoral immune responses increased with tumor progression.

One possible reason for the progression-associated increase in immune response to structural but not to nonstructural proteins was a selective increase in the expression of structural viral genes. Since the characteristic transcripts of 4.8 and 2.6 kb have not been detected in Northern blots of RNA isolated from domestic rabbit tumors, dot blot hybridizations were performed. A genomic probe gave strong signals with most tumors because of its hybridization to the E6- and E7-encoding transcripts of 2.0 and 1.3 kb, representing the major transcripts in domestic rabbit tumors. The hybridization with the structural gene probe specific for L1 and L2 mRNA was much weaker and also variable. The relative intensity of hybridization with the two probes, however, was similar for papilloma and carcinoma tissues and this suggested that there was no increase in the expression of late relative to early genes as a papilloma progressed to carcinoma. The variation observed with both types of transcripts could be a consequence of the greatly variable viral DNA content in both papilloma and carcinoma cells, which ranges from about 10 to a few hundred viral gene copies per diploid cell DNA equivalent (18, 19, 22). Some variation, although minor, may reflect a contamination of tumor tissue with normal tissue or infiltration of tumors with immune cells.

A general increase in the immune response to viral proteins during tumor progression could be explained by a more extensive interaction between virus-infected cells and the immune system at the carcinoma stage, particularly when metastases develop. However, this could not explain why the increased response is specific for the structural proteins. A possible explanation could come from in situ hybridizations. In domestic rabbit papillomas, expression of structural genes was confined to the uppermost layer of the epithelium while in carcinomas, structural genes could be detected throughout the entire tumor tissue (24). Thus, the development of carcinomas or metastases may be particularly beneficial to the immune recognition of structural proteins but may have less of an effect on the recognition of nonstructural proteins which are expressed predominantly in the lower level of the epithelium in papillomas. The finding that the immune response to the minor structural protein, L2, is almost as high as that to the major structural protein, L1, may indicate that the former is a more potent antigen. This is suggested by the finding that immunization with trpE-L2 elicits a stronger antibody response than immunization with trpE-L1 (5).

Information about the immune response with progression of tumors induced by other papillomaviruses is limited. However, available evidence suggests that changes with progression do occur with the other high-cancer-risk papillomaviruses (25). Thus, in cancer patients there was an increased response to E7 of HPV16 (4) and HPV18 (1), while a response to the late proteins was more frequently detected in patients with benign lesions associated with virus production (4). However, no differences were detected in the lymphoproliferative responses to HPV16 E4 and E6 or to HPV18 E6 between patients with HPV lesions (cervical intraepithelial neoplasias) and controls (2). In contrast, a positive lymphoproliferative response to virions or E4 was observed in individuals infected with, or with a history of infection with, the low-cancer-risk HPV1 (16). Similar to the situation with CRPV, an increased antibody response to L1 of HPV8, a high-cancer-risk cutaneous HPV, was observed in patients with squamous cell carcinoma compared with uninfected controls (17). Thus, changes in the immune response with progression of tumors associated with high-cancer-risk papillomaviruses may be a general feature and prognostic for cancer development. Here we have shown in an experimental system that progression of tumors induced by a high-cancer-risk papillomavirus is associated with an increased T-cell response to specific viral proteins.

We thank Yanan Lao, Margerita Tayag, and Xiaotao Deng for excellent technical assistance and Marcia Tryle for typing the manuscript. This research was supported by Public Health Service grant CA 50339 from the National Cancer Institute.

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