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Tumor Regression Is Associated with a Specific Immune Response to the E2 Protein of Cottontail Rabbit Papillomavirus

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Cottontail rabbit papillomavirus is the major papillomavirus animal model with which to study host–virus interactions. As with human papillomaviruses, papillomas may spontaneously regress, persist, or progress to carcinoma. Here we show that the majority (88%) of regressor rabbits had antibody to the nonstructural protein E2 compared to 29% in animals with persisting papilloma. The antibody response to other nonstructural viral proteins was the same for rabbits with regressing and persisting papilloma. The cellular immune response was measured by an *in vitro* proliferation assay. The responses to E6 and E7 were infrequent and similar in papilloma-bearing and in regressor rabbits and no rabbits responded to E1. In contrast, the response to E2 was more frequent in regressor rabbits. These data suggest that E2-specific immune responses may play a role in tumor regression.

INTRODUCTION

Papillomaviruses are small nonenveloped DNA viruses with a double-stranded circular genome of about 8 kb. Cottontail rabbit papillomavirus (CRPV) was the first papillomavirus isolated and shares with certain human papillomaviruses the feature that lesions induced by the virus progress to carcinoma. A property common to all papillomaviruses is that they can establish persistent infections in immunocompetent hosts. This can be understood, at least in part, by considering that the life cycle of the virus is strictly limited to the epithelium. Nevertheless, papillomavirus infections are subjected to some control by the immune system since immunosuppression leads to an increase in infections (reviewed in Jochmus and Altmann, 1993). Furthermore, in humans (Tagami *et al.*, 1974; Aiba *et al.*, 1986; and Tay *et al.*, 1987), as well as in animal hosts (Jarret, 1985; Campo *et al.*, 1993; Syverton, 1952; Evans *et al.*, 1962; Seto *et al.*, 1977; and Kreider, 1963), papillomas may spontaneously regress. This phenomenon has been well documented for CRPV. In an extensive study of more than 200 domestic rabbits, papillomas regressed in 9% of the animals (Syverton, 1952). In other investigations the spontaneous regression was higher, as high as 36% (Evans *et al.*, 1962). Since the domestic rabbits used were not inbred, the variations could be related to genetic differences between different rabbit populations. Evidence for this was provided by the finding that regression was linked to certain alleles of MHC II genes defined by restriction fragment length polymorphism (Han *et al.*, 1992). However, subsequent sequence analysis of the antigen binding domain of the different DRα and DQα allotypes did not reveal differences in the MHC II binding sites. This suggested that MHC II molecules were not directly responsible for the differences but rather the products of genes linked to MHC II genes (Han *et al.*, 1994).

Immunohistochemical analysis of papillomas undergoing regression showed some infiltration of leukocytes (Okabayashi *et al.*, 1991) and an accumulation of T-cells particularly in the dermis below regressing papillomas (Okabayashi *et al.*, 1993). So far there is no information about the identity of the antigen(s) recognized in spontaneous regression.

A previous analysis of the antibody response to viral proteins in 35 domestic rabbits revealed a limited response to the early proteins E1, E2, E6, and E7 and no response to E4 and E5. Most interestingly, the antibody response to structural proteins greatly increased as papillomas progressed to carcinomas (Lin *et al.*, 1993). This increase was paralleled by an increase in the *in vitro* proliferation of peripheral blood mononuclear cells (PBMCs) exposed to viral structural proteins (Selvakumar *et al.*, 1994). The group of animals analyzed previously included only four regressors, a number too small from which to derive any conclusions. In this investigation we analyzed 24 regressors and show that regressors have a much higher immune response to E2 than rabbits with progressing papillomas but are equal in their response to other viral proteins. Furthermore, PBMCs of some regressors obtained 1 month after regression proliferate *in*

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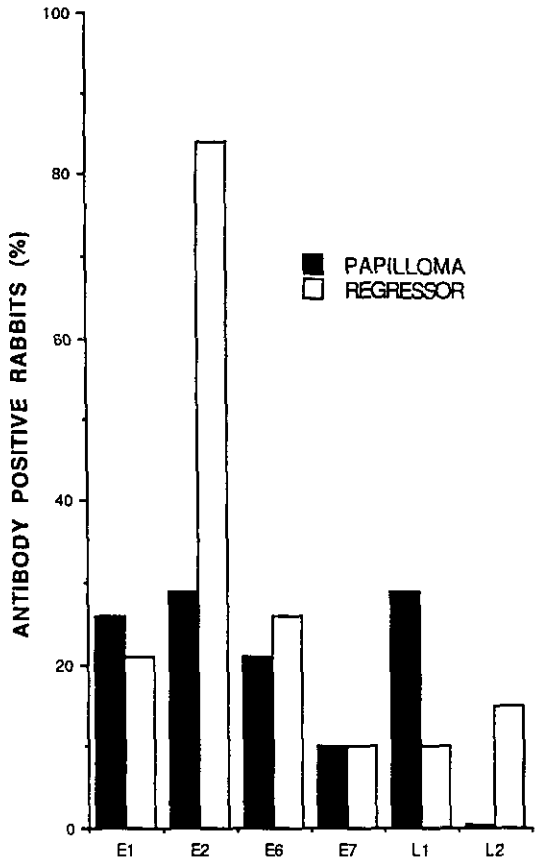

FIG. 1. Antibody response to viral proteins in rabbits with persisting and regressing papillomas. The presence of antibody to E1, E2, E6, E7, L1, and L2 was determined by Western blotting as described under Materials and Methods. The sera were obtained from 19 regressor rabbits and 34 rabbits with persisting papillomas and include 4 regressors and 31 rabbits with papillomas from a previous study (Lin *et al.*, 1993). Open bars, regressor rabbits; closed bars, rabbits with persisting papillomas.

vitro specifically in response to E2. This suggests that E2 may be a target in spontaneous regression.

MATERIALS AND METHODS

Animals and virus

New Zealand White domestic rabbits were obtained from Irish Farm, Norco, California, or from King Wheeler Farm, Ohio. Virus stocks of the Washington B strain of CRPV were prepared from cottontail rabbit papillomas as previously described (Stevens and Wettstein, 1979).

Fusion proteins

The plasmids expressing *Trp*E, *Trp*E-E6, -E7, -E2, and -E1, and the isolation of proteins as salt-insoluble fraction were as previously described (Lin *et al.*, 1993; Selvakumar *et al.*, 1994). Proteins used for *in vitro* stimulation of PBMCs were diluted to a concentration of 1–3 μg/ml with complete culture medium, consisting of RPMI 1640 (Gibco) containing 1% normal rabbit serum, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. The diluted proteins were divided into aliquots and stored at −70°.

Western blot (immunoblot) assays and immunoprecipitations

Antibodies to viral proteins were detected by Western blots as previously described (Lin *et al.*, 1993). Briefly, the blots were developed with sera blocked with *Trp*E and diluted 60-fold. All sera were tested on blots containing lanes with *Trp*E fusion protein as well as *Trp*E to verify complete adsorption of antibody reacting with *Escherichia coli* proteins. Detection of antibody to E2 by immunoprecipitation was performed using *in vitro*-translated E2 as antigen as described (Lin *et al.*, 1993).

Isolation of PBMCs and in vitro proliferation assays

PBMCs were isolated from 60–70 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-1077 (Sigma Chemical Co.), and centrifuged at 2400 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 MEM. Proliferation assays were performed in 96-well flat-bottom microtiter plates. The concentration of cells was adjusted to 2×10^5 cells per 100 μl of culture medium. The viability of the purified cells was greater than 95% as determined by exclusion of trypan blue. One hundred microliters of cell suspension and 100 μl of antigen solution were added to each well, and cultures were incubated at 37° in 5% CO_2, 95% air for 2–8 days. Additional wells contained cells and medium only or cells and 5 μg/ml concanavalin A. Twenty hours prior to harvest 1.0 μCi [^{3}H]thymidine (sp act 2 Ci/mmol; New England Nuclear Corp., Boston, MA) in 25 μl medium was added. Duplicate wells were harvested for each time point with a semiautomatic cell harvester (Flow Laboratories) and the average of the incorporated ^{3}H label was determined. To quantitate the response specific for viral antigens a stimulation index (SI) was calculated. It represents the ratio of [^{3}H]thymidine incorporated in wells with fusion protein to that in wells with *Trp*E for the same day of *in vitro* stimulation. A response is considered positive when the SI is greater than 2 (SI > 2).

RESULTS

Antibody response in regressor rabbits

In our first approach to see if regressor rabbits differed from nonregressor rabbits in the recognition of viral gene products, we analyzed their antibody response to viral proteins. In a previous analysis of the antibody response of 35 rabbits, papillomas regressed in only 4 animals, which is the average regression in our rabbit population.

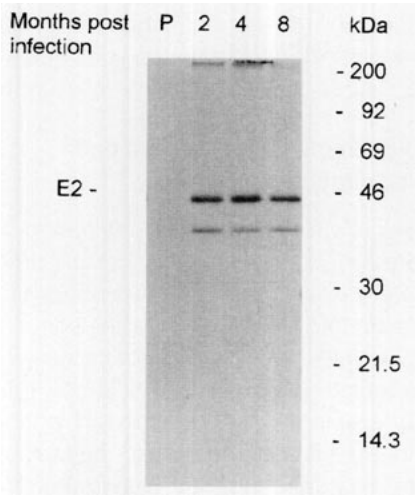

FIG. 2. Time course of the E2 antibody response in a regressor rabbit. The antibody response was followed by immunoprecipitation of *in vitro*-translated E2 as described previously (Lin *et al.*, 1993). The time the sera were obtained is indicated at the top of the lane; *P*, preinfection serum. The position of full-length E2 is indicated on the left and the positions of molecular size markers on the right.

Of the 4 regressors, 2 had antibody to E2. Although this was slightly higher than that in nonregressors, the low number did not permit any conclusion. We have now included in the analysis a total of 24 regressors. The antibody responses to E1, E2, E6, E7, L1, and L2 were examined by Western blotting using the *Trp*E fusion proteins. The results from 19 rabbits tested for antibody to all six proteins are presented in Fig. 1, which also shows the results of 34 papilloma-bearing rabbits including 31 animals previously analyzed (Lin *et al.*, 1993). The results clearly demonstrate that regressor rabbits exhibit a much higher response frequency to E2 than papilloma-bearing animals. Five additional regressor rabbits analyzed only for the presence of E2 were also all positive. When these animals are included, antibody to E2 was detected in almost 90% of the regressor rabbits compared to 29% among animals with persisting papillomas. There was no major difference between the two groups in their responses to E1, E6, E7, L1, and L2. Between 20 and 30% were positive for E1 and E6 while the response to E7 in both was about 10%. There was some difference in the responses to L1 and L2; fewer regressors responded to L1 but more responded to L2. Only selected regressors were tested for antibody to E4 and E5 but all were negative as was previously shown for rabbits with papillomas.

Since we were interested in determining whether there was a temporal link between regression and antibody response, many regressors were tested during or shortly after regression as well as months to over a year later. The results of Western blot analyses showed that at the different time intervals 3 to 4, 5 to 8, and 10 to 18 months after infection the Western blot-positive response frequency was 82, 68, and 90%, respectively. Thus, the majority of regressor rabbits remained positive during all periods. The time course of the antibody response to E2 in a regressor rabbit determined by immunoprecipitation is shown in Fig. 2. As expected the preinfection serum was negative. Two months after infection antibody to E2 could already be detected. The intensity of the E2 band remained the same in subsequent analyses. This indicates that the antibody response to E2 reached its peak during regression and remained at the same level thereafter.

Cellular immune response in regressor and nonregressor rabbits

In our previous analyses of the antibody (Lin *et al.*, 1993) and the cellular immune response (Selvakumar *et al.*, 1994) to viral structural proteins there was a parallel between antibody response and cellular immune response as both increased during progression to carcinoma. To determine if there was a unique cellular immune response to early viral proteins in regressor rabbits, PBMCs were isolated from animals at the three different stages of infection, regressors, papilloma-bear-

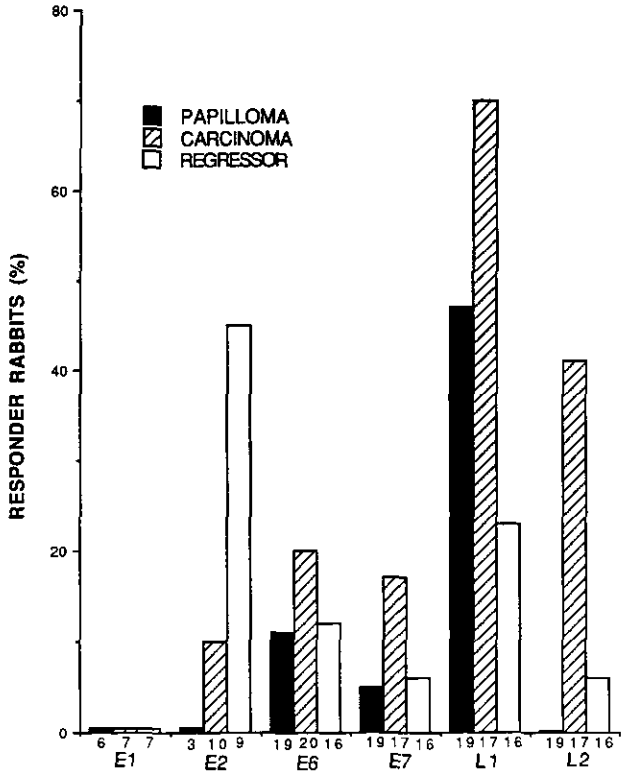

FIG. 3. Proliferative response to viral proteins at the papilloma stage, after papilloma regression, and after progression to carcinoma. The *in vitro* proliferative response was measured as described under Materials and Methods. SI greater than 2 is considered positive (Selvakumar *et al.*, 1994). Open bars, regressor rabbits, closed bars, papilloma bearing rabbits; striped bars, rabbits with carcinoma. The number of rabbits analyzed for each category is indicated below the bars. Included in the data are previously reported results of the response to L1 and L2 in 17 papilloma-bearing rabbits and 11 rabbits with carcinoma.

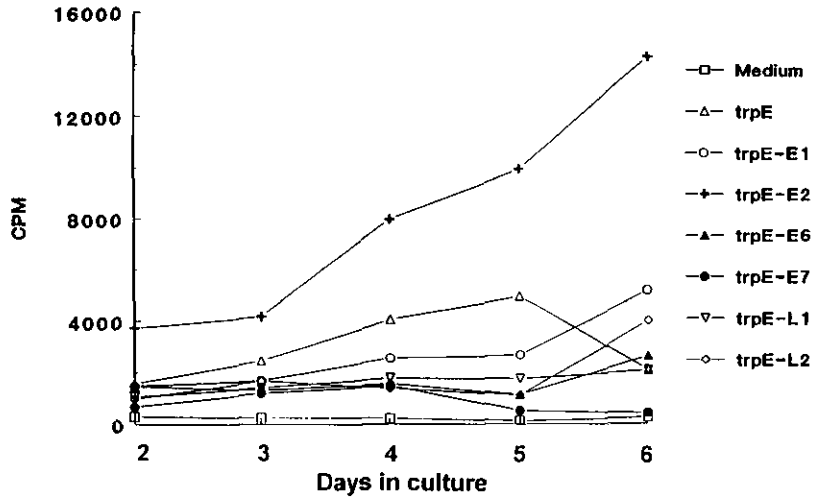

FIG. 4. *In vitro* PBMC proliferation response to CRPV fusion proteins of a regressor rabbit. The proliferation response was measured as described under Materials and Methods with PBMCs obtained 1 month after regression.

ing rabbits, and rabbits with carcinomas, and tested for their *in vitro* proliferative responses to early viral proteins E1, E2, E6, and E7. In addition, rabbits were tested for responses to the two structural proteins L1 and L2. The results of these analyses are presented in Fig. 3. The proliferative responses to E6 and E7 were determined at different time points of the different stages of infection. The responses to E1 and E2 were determined in a more limited number of animals and the majority were tested shortly after regression. Considering all measurements, two features are noteworthy. First, in none of the stages was there an *in vitro* proliferative response to E1, and second, the most frequent response in regressors was to E2.

When PBMCs obtained during regression were tested for their *in vitro* proliferative response, there was a high background proliferation in the majority of animals. Four of the seven animals with the high background were retested 1 month later when regression was complete. At this time PBMCs of all four had a specific response only to E2 and not to any other early or late protein (Fig. 4). The values of the E2 SI for these animals ranged from 2.6 to 6.8 and the data presented in Fig. 4 are for the animal with the lowest value. Two animals with a high background during regression when tested 4 months after regression did not respond to E2. This may indicate that the E2-specific proliferation response observed after regression was transient. Overall, both antibody and cellular responses point to E2 as a viral antigen targeted in regression.

DISCUSSION

Our analysis of the humoral immune response showed a high frequency of antibody to E2 unique to regressor rabbits. The cellular immune response to E2 was also detected more frequently in regressor rabbits than in nonregressors or carcinoma-bearing animals. The most compelling evidence that E2 is an antigen specifically recognized by cell-mediated immunity in regression is the finding that all four regressors tested at the end of regression were positive for this viral protein only. In regressors the antibody response to E2 was detected about twice as frequently as the proliferative response, and the antibody response persisted for over a year. Thus, immune recognition of E2 in regressors was long lived and this was previously shown to be true (Kreider, 1963; Evans and Ito, 1966) for the regression-mediating immune response.

Several lines of evidence suggest a role for the cellular immune response in papilloma regression. First, transfer of sera from animals with regressing papilloma did not increase the regression rate in recipients (Evans *et al.*, 1962). Second, regressing papilloma had a fourfold higher infiltration of leukocytes than persisting papilloma (Kreider, 1980), and T-cells were shown to accumulate in the dermis beneath regressing papillomas (Okabayashi *et al.*, 1991, 1993). Third, regressor rabbits have a higher skin reactivity than nonregressors to viral antigen (Hopfl *et al.*, 1993). Our experiments provide further support for a role of the cellular immune response in regression and suggest that E2 is an antigen recognized in this response. In contrast to the long-lived nature of the regression response, the *in vitro* proliferative response to E2 appeared to be short lived. Most likely, this reflects a limited sensitivity of the proliferation assay, permitting detection only at the response peak. Spontaneous regression of CRPV-induced papilloma is a relatively rare event but regression can be increased by immunization. Our finding that immunization with E2 increases regression (Selvakumar *et al.*, 1995) supports the notion that this antigen could play a role in spontaneous regression. In addition to E2, immunization with E1 (Selvakumar *et*

al., 1995) and E6 (Lathe *et al.*, 1989) increases regression. So far, however, we have no evidence that either of these two proteins is targeted in spontaneous regression.

Spontaneous regression appears to be a hallmark of bovine papilloma virus-4 infection in cattle. Papillomas in most animals disappear within a year (Campo *et al.*, 1993). Spontaneous papilloma regression is often observed in humans (Tagami *et al.*, 1974; Aiba *et al.*, 1986; Tay *et al.*, 1987). Interestingly, in a patient with human papilloma virus-31-associated condyloma undergoing spontaneous regression there was a transient specific *in vitro* proliferative response of PBMCs to an E7 peptide. The response was present 2 to 3 weeks after regression but was no longer detectable 3 months later (Kadish *et al.*, 1994). This is similar to the E2-specific PBMC proliferative response observed in rabbits after regression.

Our investigation of the immune response to viral proteins in regressor rabbits clearly implicates E2 as a critical antigen. The finding that the *in vitro* proliferative response can only be detected shortly after regression while the regression phenomenon appears to be long lived most likely reflects a limited sensitivity of the *in vitro* proliferation assay.

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