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Immunization with Nonstructural Proteins E1 and E2 of Cottontail Rabbit Papillomavirus Stimulates Regression of Virus-Induced Papillomas

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Cottontail rabbit papillomavirus is the major animal model for cancer-associated papillomaviruses. Here we show that vaccination with the nonstructural proteins E1 and E2 induces the regression of virus-induced papillomas and that vaccination is equally effective when proteins are given with and without adjuvant. There was no correlation between antibody levels and regression, suggesting that tumor regression may be due to a cell-mediated response.

Papillomaviruses are small DNA viruses with a double-stranded circular genome of about 8 kb. Most papillomaviruses are strictly epitheliotropic, and the viral life cycle is coupled to the differentiation of the epithelium (26). Cottontail rabbit papillomavirus (CRPV) was the first papillomavirus identified (24) and also the first DNA virus shown to be associated with the development of cancers (22). The discovery that certain human papillomaviruses are linked to cancer development (21, 31, 32) has increased the interest in CRPV as a model for cancer-associated papillomaviruses. The interaction between host and papillomaviruses is complex, with different possible outcomes (29). Papillomas may develop and persist for a long time, and with CRPV as well as with high-risk human papillomaviruses, lesions may progress to carcinomas (25). Alternately, papillomas may regress spontaneously (9, 13), and finally, infection may not lead to a visible lesion but rather to latent infection (1). The finding that papillomas spontaneously regress indicates that the immune system can recognize virus-infected cells. Furthermore, it was shown that the regression frequency could be increased by immunization with vaccines prepared from autologous or heterologous papillomas (7, 8). Thus, papillomas do contain antigens which are effective in inducing an immune response to papilloma cells. However, it is not known if the antigens are virus-encoded or virus-induced cellular proteins.

We have previously shown that immunization of rabbits with viral structural proteins L1 and L2 either in the form of fusion proteins or as recombinant vaccinia virus proteins protected rabbits against challenge with CRPV (16). Protection with L1 but not L2 (5, 15) was critically dependent on maintaining conformational epitopes (15). The protection was shown to be based on neutralization of the challenge virus, since L1-immunized animals could still be infected with DNA. Among the nonstructural proteins which deserve consideration as antigens capable of inducing an immune response causing papilloma regression are E1, E2, E6, and E7. Less likely candidates are E4 and E5, since they are poorly immunogenic in CRPV-infected rabbits (17).

Here we tested the ability of E1 and E2, the two proteins required for DNA replication (28), to induce regression of papillomas. Both proteins were tested as TrpE fusion proteins. The TrpE-E2 fusion protein vector has been described previously (17). The TrpE-E1 vector was constructed by cloning the *Taq*I fragment (nucleotides 1621 to 3416) into the *Sma*I site of pATH 23 (kindly provided by T. J. Koerner and A. Tzagoloff). Expression of the fusion proteins and their isolation were as described previously (17). Each New Zealand White rabbit received a course of three subcutaneous injections of 250 μg of protein each given at 2-week intervals. The fusion proteins were either emulsified in RIBI adjuvant (MPL + TOM + CNS emulsion; RIBI ImmunoChem Research Inc.) (16) or heat denatured and diluted in phosphate-buffered saline. Two weeks after the last booster the animals were infected at four sites by applying CRPV to lightly scarified skin. The development of papillomas was monitored over a period of 3 months, and the results are summarized in Table 1. One month after infection almost all rabbits developed papillomas, but in the majority of the fusion protein-immunized animals, the papillomas disappeared 72 days after infection. The papillomas disappeared in only 1 (8%) of the 12 TrpE-immunized control rabbits. There was no significant difference between E1 and E2 in inducing an immune response eliciting tumor regression; papillomas regressed in 13 (76%) of 17 E1-immunized rabbits, while papillomas regressed in 7 (64%) of 11 E2-immunized rabbits. Thus, both E1 and E2 were effective in eliciting an antipapilloma response.

A more detailed analysis of the appearance and disappearance of papillomas in TrpE-, TrpE-E1-, TrpE-E2-, and TrpE-E1-plus-TrpE-E2-immunized animals is shown in Fig. 1. In TrpE-immunized animals the number of positive sites reached 80% at 34 days and increased to 95% by day 58. The subsequent decrease reflects regression of papillomas in 1 of the 12 TrpE-immunized animals. In the fusion protein-immunized animals the highest percentage of positive sites was reached at 34 days and then started to decline because of the immune response. The percentage of infection sites with papillomas at 34 days was the same for TrpE-immunized control rabbits and for E1 and E2 fusion protein-immunized animals. This suggests that the immunization does not affect the ability of the

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TABLE 1. Development of papillomas in rabbits immunized with TrpE, TrpE-E1, and TrpE-E2[a]

Immunogen	Immunization	No. of papilloma-positive rabbits/ no. tested on post-infection day:		
		34	78	106
Expt 1				
TrpE	Nondenatured protein + RIBI adjuvant	6/6	6/6	6/6
TrpE-E1	Nondenatured protein + RIBI adjuvant	5/5	0/5	0/5
TrpE-E2	Nondenatured protein + RIBI adjuvant	5/5	3/5	3/5
TrpE-E1 + TrpE-E2	Heat-denatured protein	6/6	1/6	1/6
Expt 2				
TrpE	Heat-denatured protein	6/6	5/6	5/6
TrpE-E1	Heat-denatured protein	6/6	2/6	2/6
TrpE-E2	Heat-denatured protein	5/6	1/6	1/6
TrpE-E1	Nondenatured protein + RIBI adjuvant	5/6	2/6	2/6

[a] New Zealand White rabbits were immunized with different proteins and challenged with virus as described in the text.

virus to initiate an infection. The rate of papilloma regression appeared to be slightly higher in rabbits immunized with a combination of fusion proteins. Most likely the availability of a larger number of epitopes permits a stronger regression-mediating response. These data demonstrate that immunization with the early proteins E1 and E2 did not prevent virus infection but elicited a potent response to virus-infected cells.

It was noted that papillomas in immunized nonregressor rabbits seem to grow with little impairment. To quantitate the growth rate of papillomas in control TrpE- and fusion protein-immunized rabbits, the smallest and largest diameters of papillomas were measured and the area covered by the papilloma was calculated. The results of these measurements are presented in Fig. 2. No measurements were taken at the first time point (34 days) because of the small sizes. At 48 days there was a significant difference, as some papillomas were already in regression. The average growth rate of papillomas in the TrpE-immunized animals is somewhat higher than that of persisting papillomas in fusion protein-immunized animals (Fig. 2). However, the difference is statistically not significant because of a relatively large variation in size. These measurements indicate that regression after immunization is an all-or-nothing phenomenon.

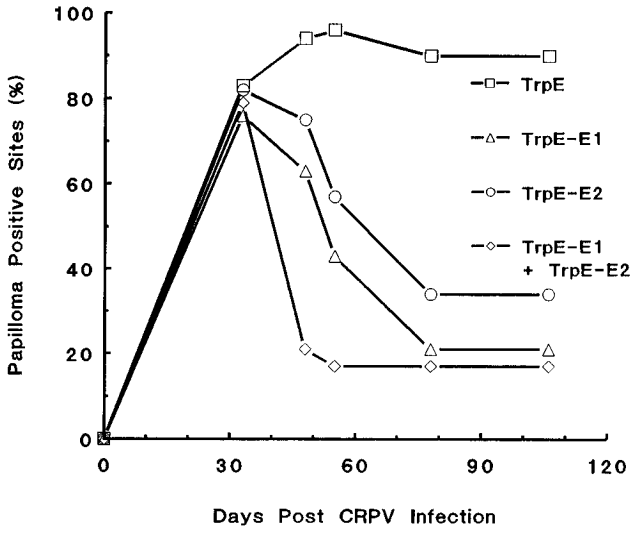

FIG. 1. Time course of papilloma development and regression in immunized rabbits. Rabbits were immunized and challenged with CRPV as described in the text and in Table 1. The data from rabbits immunized with and without adjuvant were combined, and the number of sites with papillomas was calculated as the percentage of sites inoculated on different days after infection.

In order to gain insight into a potential role of antibodies in the E1- and E2-mediated regression, the antibody status of the rabbits was determined before virus challenge. The sera were tested on Western blots (immunoblots) as described previously (17), and the results are summarized in Table 2. All animals receiving TrpE-E2 became positive for E2 independent of whether antigen was given with or without adjuvant. The frequency of an antibody response to E1, however, was higher when the antigen was given with adjuvant. In contrast to the antibody response results, there was no significant difference in papilloma regression between the two modes of vaccination with E1. In 2 of 11 animals given adjuvant, papillomas persisted, while they persisted in 2 of 6 animals immunized without adjuvant. Furthermore, of the two seronegative rabbits, one was a regressor and one was a progressor. Thus, there was no correlation between antibody production and papilloma regression.

Our results of immunization of rabbits with the early proteins E1 and E2 showed a strongly increased regression rate in animals receiving either protein. Others have shown a significantly increased regression by immunization with recombinant vaccinia virus E6 (E6 VV) but no protection with E7 VV (14). We have obtained similar results which showed that immunization with E7 VV did not provide any protection. Thus, at least three nonstructural proteins of CRPV contain epitopes which can serve as targets for an immune response to papilloma cells.

The effective protection provided by E1 and E2 and the absence of protection by E7 are surprising for two reasons. First, on the basis of S1 mapping, E7 mRNA is the most abundant mRNA in domestic rabbit papillomas while the E2 mRNA represents only a minor species (6, 20) and in situ hybridizations revealed that E1 mRNA is also a minor species (30). Second, in situ hybridization furthermore showed that E1 and E2 mRNA are predominantly expressed in the upper epithelial layers of domestic rabbit papillomas while E6 and E7 are present predominantly in the basal and parabasal layers (30). Expression of a viral protein in the lower part of the epithelium would suggest a more likely site for interaction between virus-infected cells and the immune system. Clearly, some E1 and E2 has to be expressed in the dividing cells of the epithelium in order to maintain the DNA. The finding that seroconversion, at least to E1, was not a prerequisite for regression suggests that regression is a T-cell-mediated event. The administration of the antigen as heat-denatured aggregates, rather than with adjuvant, was used as a potential means to specifically stimulate a cell-mediated immune response. This seems to have indeed occurred with E1. A redirection of the immune response from a humoral to a cellular type was also observed with hepatitis B virus surface antigen in mice when antigen aggregates were used rather than adjuvant (23). An E1- and E2-specific regression response but not an E7-specific one to cells which express little E1 or E2 but relatively large amounts of E7 suggests that E1 and E2 of CRPV but not E7 contain major epitopes relevant for regression. E7 of other

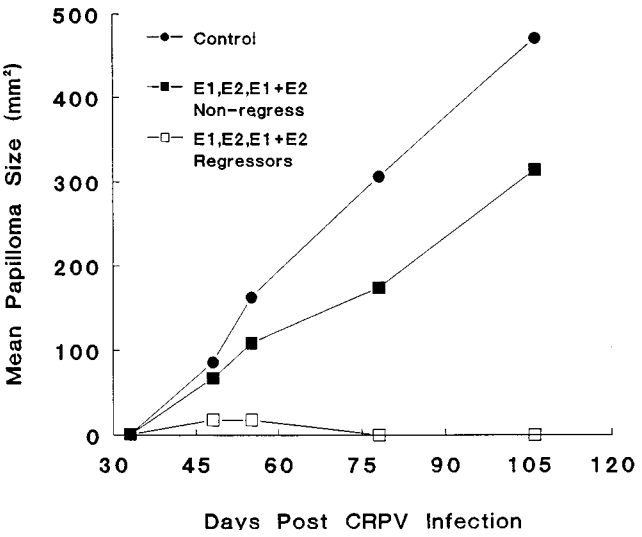

FIG. 2. Growth rate of persisting papillomas in TrpE-immunized control rabbits and fusion protein-immunized rabbits. Rabbits were immunized and challenged with CRPV as described in the text and in Table 1. The sizes of papillomas were measured on different days after infection. The size represents the area covered by the papillomas and was calculated from the diameter of the papilloma.

TABLE 2. Antibody status of rabbits after immunization with TrpE-E1, TrpE-E2, and TrpE-E1 plus TrpE-E2[a]

Immunogen	Immunization	No. of rabbits positive for antibody to protein/no. immunized[b]	
		E1	E2
Expt 1			
TrpE-E1	Nondenatured protein + RIBI adjuvant	5/5	
TrpE-E2	Nondenatured protein + RIBI adjuvant		5/5
TrpE-E1 + TrpE-E2	Heat-denatured protein	1/6	6/6
Expt 2			
TrpE-E1	Nondenatured protein + RIBI adjuvant	5/6	
TrpE-E1	Heat-denatured protein	2/6	
TrpE-E2	Heat-denatured protein		6/6

[a] New Zealand White rabbits were immunized with different proteins and challenged with virus as described in the text.
[b] The antibody status of the animals was determined by Western blotting.

papillomaviruses, most clearly E7 of human papillomavirus type 16, does contain T-cell epitopes recognized by its natural host (18, 27), and it has been shown that, at least in heterologous systems, E7 can serve as a target for a cellular immune response (3, 4, 10, 19). E2 of CRPV may function as a target not only in induced regression but also in spontaneous regression. The basis for this notion is that rabbits with regressing papillomas exhibit a heightened immune response to E2 compared with animals with progressing papillomas (unpublished data).

Papillomas in immunized rabbits have about the same growth rate as papillomas in nonimmunized rabbits; the explanation for this may lie in observations obtained with spontaneous regressor rabbits. It was shown that in spontaneously regressing animals, regression correlated with the presence of certain alleles for major histocompatibility complex type II DRα genes defined by restriction fragment length polymorphism (11). A follow-up study of this investigation, however, revealed that the restriction fragment length polymorphism was not linked to amino acid differences in the antigen binding site (12). Thus, genes other than those coding for major histocompatibility complex type II molecules may be critical for the regression.

Protection against papilloma development by early viral proteins E1, E2, E4, and E7 has been investigated with bovine papillomavirus type 4 (BPV-4) in cattle (2). In this system, protection was elicited by E7 while E2 had no effect. Since the effect of the combined immunization with E1, E2, E4, and E7 was similar to that with E7 alone it is likely that neither E1 nor E4 did play a role in tumor regression. There are similarities and differences between the CRPV and BPV-4 systems. In both systems, immunization with early proteins does not prevent infection but increases regression. A major difference is the nature of the antigen inducing regression. With BPV-4, E7 was the only early antigen which increased the regression rate while it was not effective in CRPV (14). In contrast, E2, which was definitively shown to be negative with BPV-4, is one of three early CRPV proteins found to increase the regression frequency. A second difference is that in cattle, immunization with the relevant antigen does not necessarily shorten the period over which papillomas can be observed; rather, it reduces the number of papillomas and the stage to which papillomas can develop. There is also a basic difference between CRPV and BPV-4 in the outcome of the infection in nonimmunized animals. In cattle, papillomas ultimately regress in all animals over a period of up to a year (2), while in rabbits, papillomas regress spontaneously within 2 to 3 months, but those persisting over this period rarely regress at a later stage.

The finding here that immunization with the two early proteins required for DNA replication greatly increased the regression rate of papillomas clearly demonstrates that both proteins can serve as targets for regression. A third protein which can induce such a response is E6 (14), but E7, which was considered a major candidate for such a response against human genital papillomaviruses, clearly is not effective with CRPV.

Finally, an important fact is that some rabbits with regressing tumors did not elaborate antibody to the immunizing protein while others with antibody did not regress their tumors. This clearly suggests that immune mechanisms other than a humoral response were critical; most likely it is a cellular immune response.

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